Epigenotoxicity of environmental pollutants evaluated by a combination of DNA methylation inhibition and capillary electrophoresis–laser-induced fluorescence immunoassay

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Abstract A variety of environmental pollutants may cause abnormal DNA methylation, which further disturb gene expression. In this work, we developed a rapid and sensitive method for the characterization and identification of the epigenotoxicity of environmental pollutants in terms of DNA methylation. The method combines in vitro inhibition reactions of a model DNA methyltransferase (DNMT) with rapid and sensitive capillary electrophoresis–laser-induced fluorescence (CE-LIF) immunoassays. This method was first assessed using two known DNMT inhibitors, (–)-epigallocatechin-3-gallate and RG108, and then applied to epigenotoxic evaluation of four aldehydes and six benzo-1,4-quinones. It was found that all these electrophilic chemicals could inhibit DNMT activity, probably due to their interactions with the active sites of DNMT. Interestingly, benzo-1,4-quinones displayed more inhibitory effects on DNMT activity than aldehydes. Among the tested six benzo-1,4-quinones, halogenated benzo-1,4-quinone showed higher inhibitory activity than non-halogenated *p*-benzo-1,4-quinone. Owing to its speed and sensitivity, our method will be potentially applicable for fast epigenotoxic screening of environmental pollutants and mechanistic study of environmental epigenetics.

Keywords Environmental pollutants · Epigenotoxicity · DNA methylation · DNA methyltransferase · Capillary electrophoresis–laser-induced fluorescence immunoassay

Introduction

DNA methylation, catalyzed by DNA methyltransferases (DNMTs), is one of the best characterized epigenetic modifications, which can regulate gene expression without altering the primary structure of genomic DNA. It plays crucial roles in a variety of biological processes, such as genomic imprinting, X-chromosome inactivation, regulation of chromatin organization, and cell differentiation [1–3]. Aberrant changes in DNA methylation, manifested as gene-specific hypermethylation and global hypomethylation, may disturb normal biological functions. Abnormal DNA methylation has been found in cancer as well as other human diseases [4–6], and the alteration of methylation status frequently occurs at an early stage of tumor malignant transformation [7, 8]. A number of environmental pollutants, including heavy metals and persistent organic pollutants, may directly or indirectly cause alterations of DNA methylation [9–11]. Surprisingly, the epigenetic changes induced by environmental chemicals could be heritable over several generations [12], providing a new perspective to the toxicological study of environmental pollutants [13, 14]. Identification and evaluation of the epigenetic effects and mechanisms of environmental contaminants is one of the major objectives of toxicological and biomedical research [15, 16]. On the other hand, DNA methylation may be a useful biomarker for the toxicity evaluation of environmental chemicals and early diagnosis of pollutant-induced human disease [17].

Regarding the multitude of chemicals present in the environment, fast and high-throughput methods are highly desirable for screening and characterization of their epigenetic toxicity [18]. Nuclear extract containing DNA methyltransferase

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activity has been exploited to evaluate the metal epigenotoxicity [19]. Methylation levels from the reaction system were detected by high-performance liquid chromatography/mass spectrometry. However, the time-consuming process of DNA digestion used in that method limits its application for high-throughput screening [20–22]. We have developed a rapid and sensitive method for detection of genomic DNA methylation using capillary electrophoresis (CE) coupled with laser-induced fluorescence (LIF) immunoassay [23]. By taking advantage of the immune-recognition of 5-methylcytosine in genomic DNA, global DNA methylation of diverse genomic DNA samples could be assayed by highly sensitive CE-LIF immunoassay after direct incubation with specific anti-5-methylcytosine monoclonal antibody without the need of enzymatic digestion [23].

In this work, a model DNA methylation reaction is established using pure *E. coli* CpG methyltransferase M. Sss I, which recognizes the same sequence CpG as mammalian DNMTs and has relatively stronger catalytic activity [24]. Such pure enzyme-based methylation reaction can provide explicit inhibitory information. DNA methylation levels affected by environmental chemicals are detected by CE-LIF immunoassay. Therefore, a fast and sensitive epigenetic toxicity assessment of environmental pollutants could be performed successfully by the combination of in vitro DNMT model system and CE-LIF immunoassay (Scheme 1). Effects of aldehydes and quinones on DNA methylation status are then investigated. Both types of chemicals are electrophilic, and can react with protein and DNA to form adducts [25, 26]. However, the study of their epigenotoxicology lacks. Identification of

the epigenotoxicity will be helpful for in-depth study on their pathogenic mechanisms. Taking advantage of the high speed and sensitivity, our method will be applicable for epigenotoxic evaluation and pathogenetic mechanism study of the ubiquitous environmental pollutants in terms of DNA methylation.

Materials and methods

Chemicals and reagents

RG108, (–)-epigallocatechin-3-gallate (EGCG), formaldehyde, acetaldehyde, Tetrachloro-1,4-benzoquinone (TCBQ), 2,5-dichloro-1,4-benzoquinone (2,5-DCBQ), 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2-chloro-1,4-benzoquinone (2-CBQ), 1,4-benzoquinone (p-BQ) and lambda DNA were all obtained from Sigma (St. Louis, MO, USA). 2,3-Dichloro-1,4-benzoquinone (2,3-DCBQ) was kindly provided by Dr. Benzhan Zhu (Research Center for Eco-environmental Sciences). Acrolein and crotonaldehyde were purchased from Aladdin Reagent Company (Shanghai, China). Mouse anti-methylcytosine monoclonal antibody (subclass: IgG₁) was obtained from Calbiochem (La Jolla, CA, USA). Alexa Fluor 546 labeled goat anti-mouse IgG₁ fragment Fab (secondary antibody) was supplied by Invitrogen (Carlsbad, CA, USA). *E. coli* CpG methyltransferase M. Sss I was purchased from New England BioLabs (Ipswich, MA, USA). Other chemicals in this experiment were supplied by Sigma (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA).

Scheme 1 Sketch map of our project Environmental pollutants were added to the DNMTs-mediated methylation system. Its methylation level was precipitated and methylation level was detected by CE-LIF immunoassay. Environmental chemicals with epigenotoxicity may be identified according to their inhibitory effect on the in vitro methylation system

CE-LIF Immunoassay for detection of DNA methylation

CE-LIF immunoassay was performed on the laboratory-build CE-LIF system [27, 28]. A 543.5 nm helium–neon green laser (1 mW, Melles Griot, Irvine, CA) was used for excitation and emitted fluorescence measured by photomultiplier tubes (PMT, Model R1477, Hamamatsu Photonics, Japan) at 575 nm. Methylated lambda DNA was denatured by heating at 95 °C for 5 min and then chilled on ice to prevent from DNA re-annealing. The denatured DNA was mixed with 1 µg/mL primary antibody (mouse anti-methylcytosine monoclonal antibody), and 2 µg/mL secondary antibody (Alexa Fluor 546 labeled F_{ab} fragment of goat anti-mouse IgG₁) in sample buffer (2× TGA, 14 mM Tris, 108 mM Glycine, HAc ~10.5 mM, pH 7.5). Samples were vortexed gently and incubated at 4 °C before CE-LIF analysis. Uncoated fused-silica capillaries of 25 µm i.d.×365 µm o.d. (Yongnian Optic Fiber Plant, Hebei, China) was 27 cm long with an effective length of 20 cm. The samples were electrokinetically injected into capillary by applying a voltage of 15 kV for 5 s and were separated by a voltage of 20 kV at room temperature. The buffer of 1× TG (30 mM Tris and 160 mM Glycine, pH8.5) was used as separation buffer. After each analysis, capillary was re-conditioned with 0.02 M NaOH for 5 min followed by water and running buffer for 5 min.

Method development using known DNMTs inhibitor

CpG methyltransferase M. *Sss* I (0.2 U) and 100 µM DNMTs inhibitor, EGCG or RG108, were mixed in the reaction buffer (50 mM NaCl, 10 mM Tris–HCl, 10 mM EDTA, pH 7.9). Unmethylated lambda DNA (2 µg) and S-adenosyl-L-methionine (SAM, 320 µM) were added subsequently in a final volume of 40 µL. The methylation reactions were done at 37 °C for 2 h and then inactivated at 65 °C for 15 min. The methylated DNA was precipitated with isopropanol. The pellets was washed with 70 % ethanol and re-suspended in sterilized ddH₂O. The concentration and quality of DNA were estimated by measuring the absorbance at 260 and 280 nm. The methylation of DNA sample was evaluated by CE-LIF immunoassay. Effects of DNMTs inhibitors on DNA methylation reaction were evaluated by the methylation level of lambda DNA.

Effect of aldehydes and quinones on DNA methylation

Effects of formaldehyde, acetaldehyde, acrolein, and crotonaldehyde (1,000 µM) on DNA methylation were evaluated in the reaction system as described above. Inhibitory effect of different concentrations of acrolein (0, 1, 10, 100, 1,000 µM) on DNMTs activity was also assayed.

To evaluated the effect of potential DNA damage induced by aldehydes on methylation reactions, lambda DNA (2 µg) and 100 µM acrolein were mixed and incubated at 37 °C for 2 h. Lambda DNA was then precipitated with isopropanol and washed by 70 % methanol. Purified lambda DNA was re-suspended in sterilized ddH₂O. During this process, hydrophobic acrolein could be removed. Lambda DNA reacted with and without acrolein were catalyzed by M.

Fig. 1 CE-LIF immunoassay for the inhibitory effect of DNMTs inhibitors EGCG and RG108 on methylation reaction. **a** Trace “Ab” was obtained from CE-LIF analysis of the mixture of primary and secondary antibody. Traces “EGCG”, “RG108”, and “positive control” were correspondingly obtained from CE-LIF analysis of methylated lambda DNA obtained from reaction system disturbed by EGCG, RG108 and the control group (without any inhibitor). *Peak 1* complex of primary antibody and secondary antibody; *peak 2* the immunocomplex of methylated DNA (primary antibody–secondary antibody–methylated lambda DNA). **b** Statistical result of the CE-LIF immunoassay. The detail conditions for CE-LIF immunoassays see “Materials and methods” section

Sss I at 37 °C for 2 h, and DNA methylation levels were evaluated by CE-LIF immunoassay.

Inhibitory effects of quinones on DNA methylation system were also detected using CE-LIF immunoassay. TCBQ, 2,3-DCBQ, 2,5-DCBQ, 2,6-DCBQ, 2-CBQ, and p-BQ were dissolved in acetonitrile (ACN), and added to the reaction system with the concentration of 30 μ M. Reaction system only with same volume of ACN was used as positive control. Different concentrations of 2,3-DCBQ and p-BQ (0, 0.1, 1, 10, and 100 μ M) were selected for the dose–effect relationship detection.

All the experiments were performed three times and the average was displayed in the histograms with error bars representing the standard deviation.

Results and discussion

Rapid assessment of DNA methylation inhibitory activity

To test the feasibility of our method, two known DNMT inhibitors (EGCG and RG108) were used. The interactions of the two inhibitors with *E. coli* CpG methyltransferase M. *Sss* I might inhibit the catalytic activity and reduce the degree of the methylation of DNA. EGCG, the major polyphenol component extracted from green tea, was reported to inhibit DNMT1 activity by tethering within binding site through hydrogen bonds involving Ser¹²²⁹, Glu¹²⁶⁵, Pro¹²²³, and Cys¹²²⁵ [29] and RG108, *N*-phthalyl-L-tryptophan selected through computer simulation, was identified as a human DNMT1 inhibitor [30]. To perform the DNA methylation

Fig. 2 Effect of aldehydes on DNMTs activity detected by immuno-CE-LIF. **a** Aldehydes (1,000 μ M) were added to the methylation reaction system and methylated lambda DNA was assayed by immuno-CE-LIF. The detail conditions for CE-LIF immunoassays see “Materials and methods” section. Traces “Formaldehyde”, “Acetaldehyde”, “Acrolein” and “Crotonaldehyde” were correspondingly obtained from CE-LIF analysis of methylated lambda DNA obtained

from reaction system disturbed by formaldehyde, acetaldehyde, acrolein and crotonaldehyde. *Peak 1* complex of primary antibody and secondary antibody; *peak 2* the immunocomplex of methylated DNA. **b** Statistical result of Fig. 2a. **c** Inhibitory effects of a series of acrolein with different concentrations (0, 1, 10, 100, 1,000 μ M) on methylation reaction. **d** Inhibitory effects of different concentrations of crotonaldehyde (0, 1, 10, 100, 1,000 μ M) on methylation reaction

inhibition test, DNMT inhibitor (EGCG or RG108) was added to methylation reaction system and methylation levels were assayed immediately by CE-LIF immunoanalysis.

Lambda DNA from the reaction system was incubated with primary and second antibody, and the immunocomplex of methylated DNA were detected by CE-LIF. Interestingly, only one antibody peak was observed (Fig. 1a). This is consistent with our previous work [23], which showed that the secondary antibody cannot be separated from its complex with primary antibody by free zone CE and overlapped as one peak (peak 1, Fig. 1a). Moreover, since the primary antibody has two binding sites, it is reasonable to believe that two immunocomplexes of antibody-methylated DNA can be observed. Indeed, we observed that there are two complexes of two antibodies and methylated DNA as showed by split peak 2 (Fig. 1a). The peak area ratio of peak 2 to peak 1 could be used for the accurate quantification of DNA methylation level. To display clearly the changes of DNA methylation levels, we assembled the chromatograms in one figure with the same axis, and the chromatograms were staggered in the y-axis to avoid overlap.

In contrast to the positive control (without methylation inhibitor), the intensity of the immune complex of methylated DNA in the EGCG-treated *M. Sss I* reaction decreased significantly, while that in the RG108-treated *M. Sss I* reaction also decreased but with relatively low inhibitory degree. These results suggest that the methylation of DNA is obviously inhibited by both EGCG and RG108 (Fig. 1b). The inhibitory percentages of EGCG and RG108 are about 71 and 24 %, respectively. The result confirmed that our method could be applied for in vitro epigenotoxic screening of environmental chemicals with its high sensitivity and speed.

Epigenotoxic evaluation of four aldehydes

Early studies indicated that aldehyde compounds may induce genotoxicity through the formation of DNA adducts, DNA strand breaks, DNA–DNA, and DNA–protein cross-link [26]. Formaldehyde, one of the typical aldehyde compounds, is a common indoor air pollutant, which can cause serious damage to human respiratory system and has been classified as group 1A (human carcinogen) by International Agency for Research on Cancer [31]. Toxicity of α,β -unsaturated aldehydes, such as acrolein and crotonaldehyde, are partly attributable to its high reactivity toward DNA and proteins through Michael addition reaction [32, 33]. Recent study suggested that acrolein not only induced DNA damage, but also inhibited excision repair and mismatch repair that might cause mutagenesis and initiate carcinogenesis [34]. However, it is not clear whether the reactive and electrophilic aldehydes can inhibit DNA methylation and cause epigenotoxicity.

All DNA methyltransferases have a conservative proline–cysteine dipeptide, which is known to be the most important part of the catalytic domain, and cysteine thiolate provided by the conservative dipeptide has been proved to be essential for the initiation of methyl group transfer reaction [35]. So, we hypothesized that DNA methylation level might be affected by aldehydes which could react with the proline–cysteine dipeptide at active sites of DNMTs through Michael addition reaction.

For in vitro epigenotoxicity evaluation of aldehydes, 1 mM of formaldehyde, acetaldehyde, acrolein, and crotonaldehyde were added to the methylation reaction system, respectively. The inhibition of DNMTs activity could be observed in all aldehyde-containing reactions (Fig. 2a). Compared with the positive control group, the reduction of DNA methylation level induced by acrolein and crotonaldehyde is above 90 % (Fig. 2b). Clearly, inhibition is dose dependent (Fig. 2c and d). Significant inhibition of DNMTs activity could be observed for acrolein or crotonaldehyde at a dosage of above 10 μ M (Fig. 2c and d).

It is not known whether the observed inhibition is due to the interactions of aldehydes with *M. Sss I* or due to aldehyde-caused DNA damage. To investigate the latter possibility, the effect of aldehyde-induced DNA damage on methylation reaction system is further evaluated. In such a case, lambda DNA is first treated with acrolein (100 μ M), and then the un-reacted acrolein is removed during the process of DNA purification. In such a treatment, it is supposed that the DNA damage in the treated lambda

Fig. 3 Effect of acrolein-induced DNA damage on methylation reaction system was evaluated for the methylation inhibition mechanism investigation. Trace 1 was obtained from immuno-CE-LIF analysis of methylated lambda DNA. Trace 2 was obtained from CE-LIF immunoassay of methylated lambda DNA which was reacted with acrolein before catalyzed by *M. Sss I*. *Peak 1* complex of primary antibody and secondary antibody; *peak 2* the immunocomplex of methylated DNA

DNA can be induced by acrolein. The readily damaged lambda DNA is further methylated by M. Sss I. Interestingly, no inhibition on DNA methylation is observed. Compared with lambda DNA that is not reacted with acrolein (assumed as 100 %), no significant difference is detected in methylation level of the lambda DNA pretreated with acrolein (about 99 %) (Fig. 3). Our results indicate that the potential DNA damage caused by acrolein and other aldehydes cannot affect the methylation reaction obviously. Therefore, the observed inhibitory effect of acrolein on enzyme-catalyzed DNA methylation may mostly attribute to the reaction between aldehydes and the active sites of DNMTs.

Epigenotoxic evaluation of quinones

We further tested the inhibitory effect of quinones on DNA methylation as the second example. Quinones were found to cause multiple hazardous effects, e.g., immunotoxicity, cytotoxicity and carcinogenesis [25, 36]. 2,6-dichloro-1,4-

benzoquinone (DCBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone, and 2,6-dibromo-1,4-benzoquinone have been detected as new disinfection by-products in drinking water recently [37, 38]. Quinones were reported to be able to react with thiol functional groups via Michael-type addition, so we hypothesized that the free cysteine residues of DNMTs located in their catalytic center may be modified by these compounds, leading to inhibition of their activities. Indeed, all the tested quinones, TCBQ, 2,3-DCBQ, 2,5-DCBQ, 2,6-DCBQ, 2-CBQ, and p-BQ, showed the significant inhibition on DNA methylation reaction when 30 μ M quinones were added in the reaction system (Fig. 4a). The observed inhibitory potency of the tested quinones is higher than the tested aldehyde compounds (Fig. 4a vs Fig. 2a). Although quinones may cause damage to DNA, the formation of the oxidation DNA damage is very low (<0.1 %). The observed inhibition is reasonable to be attributed to the interaction of quinones with the DNMT.

DNA methylation reaction can be disturbed by different concentrations of 2,3-DCBQ (0, 0.1, 1, 10, and 100 μ M) as

Fig. 4 Effect of quinones on methylation reaction detected by immuno-CE-LIF. **a** Methylated lambda DNA precipitated from reaction system which contained 30 μ M quinones was assayed by immuno-CE-LIF. The detail conditions for CE-LIF immunoassays see experimental section. Traces “TCBQ”, “2,3-DCBQ”, “2,5-DCBQ”, “2,6-DCBQ”, “2-CBQ” and “p-BQ” were correspondingly obtained from CE-LIF analysis of methylated lambda DNA obtained from reaction

system disturbed by TCBQ, 2,3-DCBQ, 2,5-DCBQ, 2,6-DCBQ, 2-CBQ and p-BQ. **Peak 1** complex of primary antibody and secondary antibody; **peak 2** the immunocomplex of methylated DNA. **b** Statistical result of Fig. 4a. **c** Inhibitory effects of different concentrations of 2,3-DCBQ (0, 0.1, 1, 10, 100 μ M) on methylation reaction. **d** Inhibitory effects of a series of p-BQ with different concentrations (0, 0.1, 1, 10, 100 μ M) on methylation reaction

detected by CE-LIF immunoassay. Significant decrease of methylation level (about 75 %) is detected even treated with 2,3-DCBQ as low as 0.1 μM , while decrease is not significant when treated by 2,3-DCBQ at lower concentration and the reason is not clear (Fig. 4c). Inhibitory effect of p-BQ (0, 0.1, 1, 10, and 100 μM) on DNMTs activity is also observed and the reaction system is disturbed obviously when 10 μM of p-BQ was added (Fig. 4d). Compared with un-substituted p-BQ, halogenated quinones exhibited higher inhibitory effects (Fig. 4c and d), indicating the halogenation of quinones can enhance the inhibitory effects of quinones on DNA methylation reactions.

Based on our data, we may speculate that quinones may induce aberrant DNA methylation in vitro as well as in vivo through the inhibition of DNMTs activity in catalyzing cytosine methylation. By combining the in vitro methylation reaction and CE-LIF immunoassay, inhibitory effect of various quinones on DNMTs activity could be evaluated sensitively.

Epigenotoxic evaluation of electrophilic compounds (as demonstrated by aldehydes and quinones) may provide a new approach for elucidation of the toxic mechanism and will be helpful for the control and early diagnosis of electrophilic compounds-induced human disease. In addition, by combining with 96 array capillary electrophoresis, our method may provide the high-throughput analysis, and it will be useful for large-scale screening and in-depth studies of epigenotoxic effects of the ubiquitous environmental pollutants.

In summary, highly sensitive CE-LIF immunoassay is developed for the epigenotoxicity evaluation of environmental chemicals in combination with M. Sss I-mediated DNA methylation reaction as a model system. With the high speed, sensitivity and specificity of CE-LIF immunoassay, our method will be helpful for detection of aberrant DNA methylation induced by environmental pollutants. Our study may suggest the inhibitory effects of quinones and aldehydes on DNMTs activity. Our method will be useful for the epigenotoxic evaluation of environmental pollutants and the epigenetic mechanism study of environmental-related human diseases.

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