Biochemical and Biophysical Research Communications 397 (2010) 397–400

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

SiO_2 nanoparticles induce global genomic hypomethylation in HaCaT cells

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ARTICLE INFO

Article history:
Received 23 April 2010
Available online 23 May 2010

Keywords:
Nano-silicon dioxide (nano-SiO_2)
DNA methylation

ABSTRACT

The increasing amount of nanotechnological products, found in our environment and those applicable in engineering, material sciences and medicine has stimulated a growing interest in examining their long-term impact on genetic and epigenetic processes. We examined here the epigenomic response to nm-SiO_2 particles in human HaCaT cells and methyltransferases (DNMTs) and DNA-binding domain proteins (MBDs) induced by nano-SiO_2 particles. Nm-SiO_2 treatment induced global hypoacetylation implying a global epigenomic response. The levels of DNMT1, DNMT3a and methyl-CpG binding protein 2 (MBD2) were also decreased in a dose dependent manner at mRNA and protein level. Epigenetic changes may have long-term effects on gene expression programming long after the initial signal has been removed, and if these changes remain undetected, it could lead to long-term untoward effects in biological systems. These studies suggest that nanoparticles could cause more subtle epigenetic changes which merit thorough examination of environmental nanoparticles and novel candidate nanomaterials for medical applications.

1. Introduction

Due to the unique physical and chemical characteristics, nanomaterials are becoming widely used. However, the potential toxic effects of nanomaterials are drawing attention to toxicologists. As a new type of nano-powder materials, nano-silicon dioxide (nano-SiO_2) has been widely used in a number of fields including plastic, rubber, ceramics, coatings, adhesives and so on [1]. Nano-SiO_2 particles can be readily evaporated into air due to their very low density. Previous studies have demonstrated that inhalation of SiO_2 nanoparticles could cause pulmonary and cardiovascular damage in old rats, such as pulmonary inflammation, myocardial ischemic damage, atrio-ventricular blockage, and increase in fibrinogen concentration and blood viscosity [2]. Recently, we found that nano-SiO_2 could induce cytotoxicity and protein alterations in HaCaT cells [3]. Nanoparticle or metal ion-induced oxidative stress leading to cell death has been previously reported at the genomic level, but has not yet been investigated at the epigenomic level [4]. Our previous study also showed that nano-SiO_2 can induce oxide stress, genomic stress; however, whether nano-SiO_2 can induced the change of epigenetic change is not clear.

In the present study, we compared the global DNA methylation profiles in HaCaT cells induced by nm-SiO_2 particles. We also investigated the dynamic alterations of some methylation-associated enzymes during this process. Here, we report that besides causing direct DNA damage, nm-SiO_2 particles can also induce epigenetic changes in HaCaT cells.

2. Materials and methods

2.1. Materials

15-nm SiO_2 as used in our previous study [3] was purchased from Wan Jing New Material Co. Ltd. (Hangzhou, Zhejiang, China), and micro-sized SiO_2 (1–5 μm) and 5-aza-deoxycytidine (DAC) were supplied by Sigma–Aldrich (Sigma, SL, USA). Human epidermal keratinocyte cell line HaCaT was purchased from China Center for Type Culture Collection (Wuhan, Hubei, China). MEM culture media were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Fetal bovine serum (FBS), penicillin–streptomycin for cell culture and trypsin were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Mouse monoclonal antibodies against 5-mC (Oxford, UK). Goat anti-mouse Ig(H+L)-FITC secondary antibody was purchased from Southern Biotechnology, Inc. (Anaheim, AL, USA).

2.2. Cell culture and the treatment with SiO_2 particles

HaCaT cells were cultured in MEM media containing 10% FBS, 5% carbon dioxide (CO_2) at 37 °C. SiO_2 particles of different concentrations were administered when the cell confluence reached up to 80%, and the cells were treated for 24 h. The final concentrations of

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doi:10.1016/j.bbrc.2010.05.076

SiO_2 particles were 2.5, 5, 10 μg/mL nm-SiO_2 particles, 10 μg/mL micro-SiO_2 particles, For the DNA methyltransferases inhibition assay, 5-aza-deoxycytidine (DAC, St Louis, MO) was treated to 10 μg/mL nm-SiO_2 cells at the concentration of 3 μM for 48 h.

2.3. Immunofluorescence assay

The immunofluorescence assay to measure the formation of 5-methylcytosine was performed as reported previously [5].

2.4. Flow cytometric analysis of methylated DNA

Briefly, cells were trypsinized, pelleted by centrifugation, and washed in PBS (Ca^{2+} and Mg^{2+} free). Dissociated cells were fixed and immunolabeled as previously described by Giraldo et al. [6].

2.5. High performance capillary electrophoresis (HPCE) assay

DNA extraction was carried out with tissue DNA extraction kit (Omegabiotek, Alexa, USA). RNA digestion was performed by adding 20 mg/mL RNAase A (Invetrogen, CA, USA) and incubating the mixture at 37 °C for 30 min. Precipitation of genomic DNA was performed with 1/10 volume of 3 M sodium acetate and two volumes of cold ethanol and the resulting pellet was washed with cold 70% ethanol. Genomic DNA was resuspended in Milli-Q grade water (0.5 μg/μL) and stored at 4 °C.

DNA samples (10 μL, 0.5 μg/μL) were heated for 3 min in a boiling water bath and cooled rapidly in ice; 1.5 μL of 10 mM $ZnSO_4$ and 1.5 μL of nuclease P1 (200 units/mL in 30 mM $C_2H_3O_2Na$) were added and mixtures were incubated for 16 h at 37 °C. 1.5 μL of Tris (0.5 M, pH 8.3) and 1.5 μL of alkaline phosphatase (50 units/mL in 2.5 M $(NH_4)_2SO_4$) were then added and mixtures were incubated for an additional 2 h at 37 °C. Samples were centrifuged and stored at 4 °C. The mC content was determined by Waters capillary ion analyzer (waters, MA, USA).

2.6. Real-time RT-PCR

Total RNA was extracted by Trizol Reagent (Invitrogen, AL, USA). The cDNA was synthesized from 2 μg of total RNA using PrimeScript™ RT reagent Kit (Takara, Dalian China). Quantitative PCR was performed using SYBR®Premix Ex Taq™ (Takara, Dalian China) and an MX4000 Cycler Thermal Cycler (Bio-Rad Laboratories, CA, USA). The number of cDNA molecules was normalized to that of ACTB. PCR amplification was carried out using the sets of primers designed by primer 5 (Table 1).

2.7. Western blotting

Cells were lysed in 2D lysis buffer (10 mM Tris–HCl, pH 7.5, 7 M Urea, 2 M Thiourea, 4% CHAPS, 2 M Thiourea) with a protease inhibitor cocktail III (Sigma, SL, USA). Protein samples were subjected to western blotting using antibodies (Santa Cruz, CA, USA) to DNMT1, DNMT3a and MBD2. The bands were visualized after incubation with chemiluminescent substrates (Thermo scientific, IL, USA).

Table 1
The primer sequences of related genes.

Gene	Primer sequence (5′————3′)		Annealing temp (PCR cycle no)
DNMT1	F	ACGACCCTGACCTCAAATAT	60 °C (40)
	R	CCATTAACACCACCTTCAAG A	
DNMT3a	F	CACAGAAGCATATCCAGGAG	60 °C (40)
	R	CACATTCTCAAAGAGCCAGA	
DNMT3b	F	AGTATCAGGATGGGAAGGAG	60 °C (40)
	R	CGATAGGAGACGAGCTTATTG	
MECP2	F	GTTAGGGCTCAGGGAAGAAA	60 °C (40)
	R	CCTGACCCTTCTGATGTCTC	
MBD2	F	ACTATAAGTGCCCTCTGTGT	58 °C (40)
	R	TCAGAGTCTCCTTCATGTACTT	

2.8. Statistics

Statistical analysis was performed using ANOVA. Data were expressed as mean ± SD. Differences with a P value less than 0.05 were considered to be statistically significant.

3. Result

3.1. Global DNA hypomethylation is accompanied HaCaT cells induced by nm-SiO_2 particles

Global DNA methylation was evaluated based on the mean density of green fluorescence which was assessed with a specific monoclonal antibody against 5-methylcytosine. The global DNA methylation level decreased gradually with increased dose of nm-SiO_2 in normal HaCaT cells (Fig. 1A and B). When cells induced by 10 μg/mL nm-SiO_2 particle, the mean density of fluorescence was reduced to 55.16, while 153.43, 76.32, 53.26 for control, 2.5 and 5 μg/mL groups, respectively. There was no statistically significant difference between control and micro-SiO_2 groups. Immunofluorescence assay supported the result. To verify the results further, we performed HPCE assay (Fig. 1C) to quantitate the methylated CpG% which reflects the genome methylation level. An average proportion of methylated mC/C was 4.82% in control, 2.7% in 2.5 μg/mL and 2.17% in 10 μg/mL groups, while 3.1% in micro-SiO_2 groups, which got the consistent downtrend of genome methylation level during increasing nm-SiO_2 dose nanoparticles.

3.2. DNA methyltransferases coordinate with MBDs in regulating genome methylation during cellular senescence

We further examined the expression of methyltransferases and MBDs in different groups. As shown in Fig. 2A, the mRNA expression level for DNMT1 and DNMT3a decreased gradually with increased dose of nm-SiO_2 nanoparticles. The mRNA expression of MBD2 changed in a trend similar to that of DNMT1 and DNMT3a in the process of SiO_2 treatment. The alterations at protein level were similar to those at the mRNA level (Fig. 2B). Furthermore, DAC, a DNA methyltransferase inhibitor, reduced the expression of DNMT1 and DNMT3a at a concentration of 3 μM (Fig. 2A and B).

4. Discussion

Our work contributes the first genome-wide study of DNA methylation in HaCaT cells induced by nm-SiO_2 particles. We find that nano-SiO_2 and micro-sized SiO_2 exposure could significantly decreased genomic DNA methylation and the related methyl transferase.

Increasing lines of evidence indicate that many nanomaterials have potential toxicity due to their unique physical–chemical properties [7]. HaCaT is an immortalized epithelial cell line from adult human skin that exhibits similar biological properties to normal human keratinocyte, and is an ideal cell model for studying dermal toxicity [8]. Previous study suggested that nano-SiO_2 exposure also results in DNA damage [9], size-dependent hydroxyl radicals generation [10] and lung fibrogenesis in rats [11]. Skin is a potential primary route of occupational dermal exposure for nanometer materials.

This study is only an initial step in the unexplored field of epigenetic changes by nanoparticles, which goes far beyond the

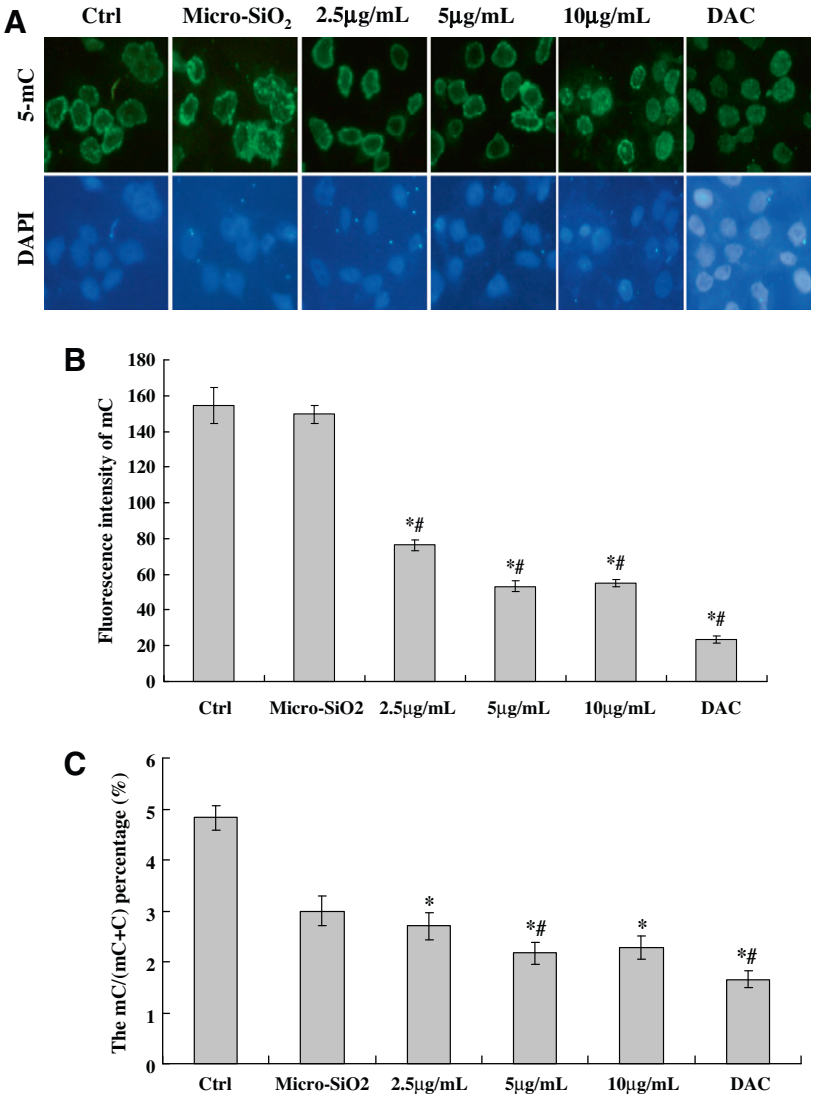

Fig. 1. Analysis of global DNA methylation in HaCaT cells induced by SiO_2 particles (A) Anti-5-methylcytosine antibody immunofluorescence. The nuclei were counterstained with DAPI (blue). The green represents anti-5-methylcytosine antibody immunofluorescence. (B) Quantitative representation of fluorescence mean density of 5-mC determined by flow cytometry assay. (C) Quantitative result of mC/(mC+C) by HPCE. Each point represents the mean ± SD in three independent experiments. $^{*}p < 0.05$ versus control cells; $^{\#}p < 0.05$ versus cells exposed to 10 μg/mL standard SiO_2. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

assays employed here. DNA methyltransferases (DNMTs) are responsible for maintaining methylation status in the genome, and catalyze the transfer of a methyl moiety from *S*-adenosyl-L-methionine (SAM) to the cytosine of a CpG dinucleotide. The mammalian DNMTs family is divided into maintenance and de novo methyltransferases, including DNMT1, DNMT3a and DNMT3b. Methyl-CpG binding protein 2 (MeCP2) and methyl-CpG binding domain (MBD2), the founding members of the DNA-binding domain proteins (MBD) family, bind to methylated DNA and suppress transcription from a methylated target gene, promoting interactions with other proteins [12].

Therefore, DNA methyltransferases cooperatively regulate cytosine methylation, providing an epigenetic basis for gene silencing and maintenance of genome integrity, while MeCP2 and MBD2 might act as the 'platform' for methylation or as the compensation to global hypomethylation. In summary, DNMT1, DNMT3a and MBD2 play important roles in HaCaT cells induced by SiO_2 particles.

Epigenetic changes induced by nanoparticles similar to those described in the present studies merit further investigations addressing the question of how nanoparticles impact on the epigenome in many other cells in vitro, and more importantly, in vivo. With the growing array of nanotechnological products, it is important to identify key factors and establish reliable tests, including epigenetic screening ("nanoepigenetics") to predict toxicity and provide guidance for creating safe nanomaterials [13].

In conclusion, our study has found evidence of early epigenetic dysregulation in HaCaT cells that may mediate, in whole or in part, the long-term consequences SiO_2 induced cell toxicity. Epigenetic dysregulation may have long-term effects on gene expression programming long after the initial signal has been removed, and if these changes remain undetected, it could lead to long-term untoward effects in biological systems. By using a genome-wide approach, we have uncovered reproducible and validated changes in cytosine methylation that occur in HaCaT cells. While the direct causality and subsequent physiological consequences of these altered epigenetic states remain unclear, this genome-wide approach to studying cytosine methylation provides a novel means of gaining insights into the mechanism of toxicity of nanomaterials.

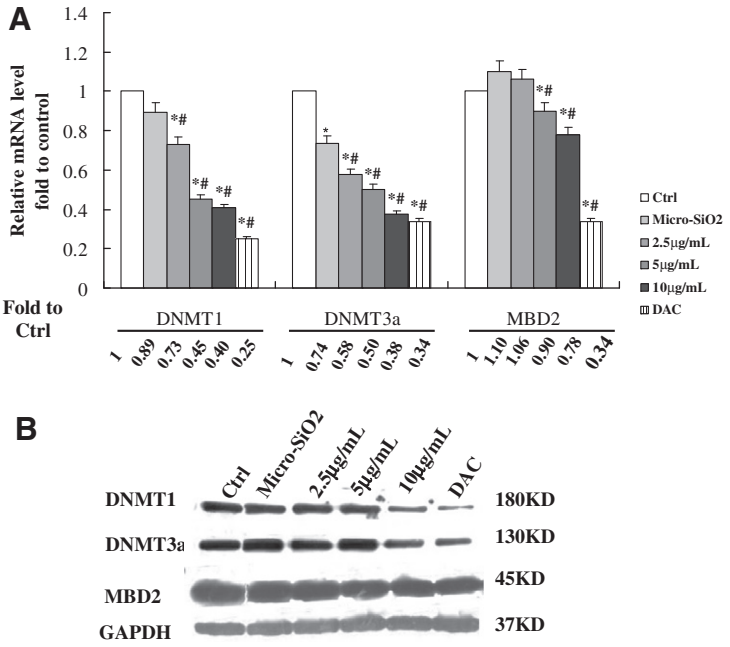

Fig. 2. Differential expression of DNMTs and MBDs (A) Levels of mRNA for DNMT1, DNMT3a and MBD_2. Data were expressed as the mean ± SD in three independent experiments, representing the relative levels with normalization by ACTB. The fold differences were calculated compared with control groups and shown in the figures. (B) Protein levels were detected by immunoblotting with specific antibodies against DNMT1, DNMT3a and MBD_2 in different groups. GAPDH was included as an internal control.

Conflict of interest

None of the authors has any potential conflict of interest or financial interests to disclose.

Acknowledgments

This work was supported by the National Natural Science Foundation of China [30972505] and Shenzhen Science Technology Plan Key Project [200901017].

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.05.076.

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