Biochemical and Biophysical Research Communications 414 (2011) 252–258

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

The involvement of DNA and histone methylation in the repression of IL-1β-induced MCP-1 production by hypoxia

Yoko Aoi [a,b], Ken-ichi Nakahama [a], Ikuo Morita [a,b,*], Olga Safronova [a,b]

[a] Department of Cellular Physiological Chemistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
[b] Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan

ARTICLE INFO

Article history:
Received 12 September 2011
Available online 17 September 2011

Keywords:
Hypoxia
Monocyte chemoattractant protein-1 (MCP-1)
DNA methylation
Histone methylation
Transcriptional repression
Inflammation

ABSTRACT

Hypoxia is a microenvironmental pathophysiologic factor commonly associated with tumors and tissue inflammation. We previously reported that hypoxia repressed IL-1β-induced monocyte chemoattractant protein-1 (MCP-1) expression. The purpose of this study was to investigate the mechanisms involved in the repression of MCP-1 expression under hypoxia. Treatment of HeLa cells with 5-aza-dC, an inhibitor of DNA methylation, abolished the repression of IL-1β-induced MCP-1 expression by hypoxia. A detailed study of the methylation of CpGs sites using bisulfite-sequencing PCR and 5-methylcytosine immunoprecipitation showed that hypoxia induced DNA methylation in both the enhancer and promoter regions of MCP-1in IL-1β-treated cells. Next, we analyzed histone methylation within the MCP-1 promoter and enhancer regions. The level of H3K9 di-methylation, a mark of gene repression, in both promoter and enhancer regions was increased by hypoxia in IL-1β-treated cells. Our findings suggest that changes in the methylation status of CpGs, as well as histone 3 methylation, may represent a critical event in transcriptional repression of IL-1β-induced MCP-1 expression by hypoxia. Therefore, DNA methylation is associated with not only epigenetic gene silencing, but also with transient transcriptional repression.

1. Introduction

Tissue hypoxia occurs as a result of inadequate blood flow to tissues or increased oxygen consumption. Hypoxic conditions have been shown to be associated with various pathologies like cancer, inflammation and wounds as well as stem cell maintenance and development [1]. Within normal cells, severe hypoxic stress generally leads to cell cycle arrest, apoptosis, and necrosis [2]. However, tumor cells, which often have areas of hypoxia, can survive and proliferate in this adverse environment by inducing genes that increase angiogenesis and glycolysis [2]. Stress-induced changes in gene expression are mediated by changes in chromatin structure. Recently, the transcriptional responses to hypoxia have been extensively studied; however, less attention has been paid to the epigenetic mechanisms of gene repression during hypoxia [3].

Post-translational modifications of histone tails, such as acetylation, methylation, ubiquitination, and phosphorylation, and DNA methylation are important for chromatin remodeling and gene transcription [4]. Specific modifications of histone tails can be divided into those that correlate with transcriptional activation and those that correlate with transcriptional repression. In general, acetylation of histone tails is associated with actively transcribed genes [4]. Methylation of lysine residues within histone tails may have either positive or negative effects on transcription depending on the site, for example, methylations on histone H3 lysine 4 (H3K4) and histone H3 lysine 36 (H3K36) are often associated with transcriptional activation, whereas methylations on histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) correlate with transcriptional repression [5].

One of the critical mechanisms of epigenetic regulation depends on the methylation of cytosines within CpG sites of gene regulatory sequences. Methylation of CpG dinucleotides is generally associated with transcriptional silencing and is maintained through cell division [6]. DNA methylation mediates transcriptional repression by interaction of the methylated cytosines with methyl-binding proteins. These subsequently recruit multi-protein complexes containing histone deacetylases and other co-repressors and modify the chromatin structure [7]. DNA methylation has been considered to be involved in heterochromatin formation in development, genomic imprinting and X chromosome inactivation [8,9]. Although it has been thought that DNA methylation is a highly stable silencing marker that is not easily reversed [10], recent studies have shown that transient DNA methylation is caused by various stimulations [11–13]. Cyclical methylation and demethylation of CpG dinucleotides were observed in gene regulation in human cells

* Corresponding author at: Department of Cellular Physiological Chemistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan. Fax: +81 5803 0212.
E-mail address: morita.cell@tmd.ac.jp (I. Morita).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.09.066

upon activation by estrogen or doxorubicin [14,16]. Recently, Kim et al. [12] proved that DNA methylation/demethylation is hormonally switched to control transcription of the CYP27B1 gene.

In this study, we focused on DNA and histone methylation and addressed the involvement of these modifications in IL-1β-induced MCP-1 gene expression by hypoxia. Our results demonstrated that methylation of DNA and histone H3K9 could be attributed to transcriptional repression of IL-1β-induced MCP-1 expression by hypoxia. These data provide new evidence that DNA methylation may be associated with transient transcriptional repression.

2. Materials and methods

2.1. Cell culture and hypoxia treatment

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. A hypoxic gas mixture containing 5% CO_2, 0.5% O_2, balanced with N_2 was used for hypoxia treatment. Long-term hypoxia (2 or 6 h) was achieved with a modular incubator chamber (Billups-Rothenberg, Inc.). O_2 concentrations were routinely measured using an Oxygen Monitor JKO-25S (Jiko, Japan). Short-term hypoxia (15 or 30 min) was achieved by bubbling a hypoxic gas mixture to the medium for 15 min. For 1 h hypoxia treatment, the hypoxic-conditioned medium was used in the modular incubator chamber. Treatment with or without 50 ng/ml IL-1β (PeproTech) was performed for the indicated time periods.

2.2. Immunocytochemistry

Immunocytochemical assays were performed using the standard procedure. To ensure that methylated DNA was accessible to antibodies, the DNA was denatured with 2 M HCl with subsequent neutralization in 100 mM Tris/HCl. Incubation with an anti-5-methylcytosine (EPIGENTEK) antibody was followed up by Alexa Fluor 488-conjugated IgG (Invitrogen). Propidium Iodide (Nacalai Tesque) was used to stain nuclei. Images were visualized using a confocal microscope (Zeiss LSM 510, Carl Zeiss, Jena, Germany).

2.3. DNA methyltransferase (DNMT) and DNA demethylase activity assays

Nuclear proteins were extracted using an EpiQuik Nuclear Extraction Kit (EPIGENTEK). Protein concentrations were measured with a Bio-Rad protein assay reagent (Bio-Rad). DNMT activity and DNA demethylase activity were measured in HeLa nuclear extracts using EpiQuik DNMT Activity/Inhibition Assay Kit or EpiQuik DNA Demethylase Activity/Inhibition Assay Kit (EPIGENTEK) in accordance with the manufacturer's protocol.

2.4. Treatment with 5-aza-2′-deoxycytidine

Cells were seeded at low density. To block DNA methylation, 24 h later cells were treated with 2.5 μM 5-aza-dC for 48 h, and the medium was changed every 24 h. Following incubation with 5-aza-dC, cells were exposed to hypoxia in combination with or without IL-1β for 6 h.

2.5. Quantitative real-time PCR (Q-PCR)

Total RNA was isolated from cultured HeLa cells using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA synthesis was performed for 1 h at 42 °C using ReverTra Ace kit (TOYOBO) with 5 μg of total RNA as a template.

Quantitative PCR was performed in a LightCycler™ instrument (Roche Diagnostics) using fluorescence resonance energy transfer (FRET) hybridization probes with LightCycler FastStart DNA Master^{PLUS} HybProbe (Roche). The primer and probe sequences were as follows:

Human MCP-1 (GenBank ID: BC009716).
Sense, 5′-ATGCAATCAATGCCCCAGTC-3′;
Antisense, 5′-TGCAGATTCTTGGGTTGTGG-3′;
LC-probe, 5′-GCTGTGATCTTCAAGACCATTGTGGC-3′;
Flu-probe, 5′-CACCAGCAGCAAGTGTCCCAAAGA-3′.
Human 18S rRNA (GenBank ID: X03205).
Sense, 5′-GTGATGCCCTTAGATGTCC-3′;
Antisense, 5′-CCATCCAATCGGTAGTAGC-3′;
LC-probe, 5′-TTCCCAGTAAGTGCGGGTCATAAGCT-3′;
Flu-probe, 5′-TGCAATTATTCCCCATGAACGAGGA-3′.

cDNA from the reverse transcription step was diluted 10 times (MCP-1) or 1000 times (18S rRNA) and 2 μl of dilution mixture was pipetted into each capillary. As a negative control, 2 μl of PCR-grade water was added.

2.6. Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed using a Chromatin Immunoprecipitation Assay Kit (Millipore) in accordance with the manufacturer's protocol with the following exceptions: cross-linking was performed at room temperature by adding formaldehyde for 10 min with subsequent termination with glycine at a final concentration of 0.125 M for 5 min. Chromatin was sheared using a sonicator Bioruptor UCD-200T (Cosmo Bio Co., Ltd.). Chromatin from 3×10^6 HeLa cells was used for each immunoprecipitation reaction with one of the following antibodies: anti-5-methylcytosine (EPIGENTEK), anti-H3K4me3 or anti-H3K9me2 (Millipore). Samples were analyzed by Q-PCR in a LightCycler™ instrument using a DyNAmo Probe qPCR kit (Thermo Scientific). TaqMan probes were labeled with a 5′-FAM reporter and 3′-BHQ1 non-fluorescent quencher. The primer and probe sequences were as follows:

MCP-1 enhancer,
Sense, 5′-GGCCCAGTATCTGGAATGCA-3′;
Antisense, 5′-TCAGTGCTGGCGTGAGAGAA-3′;
Probe, 5′-TTCTCTTCTACGGGATCTG-3′;
MCP-1 promoter,
Sense, 5′-AATCCACAGGATGCTGCATTT-3′;
Antisense, 5′-GGCTGCTGTCTCTGCCTCTT-3′;
Probe, 5′-CTCAGCAGATTTAACAGC-3′.

For Q-PCR, 2 μl from a total 40 μl DNA extracted after immunoprecipitation were used. After an initial denaturation step (50 °C for 2 min, 95 °C for 10 min) amplification was performed for 40 cycles (95 °C for 15 s, 60 °C for 60 s). Immunoprecipitated DNA was normalized by 10% input DNA. Data are expressed as a percentage of histone methylation at 0 min. The pGL3-Basic Vector (Promega) containing the MCP-1 promoter region was used to obtain the calibration curve.

2.7. Bisulfite sequencing PCR (BSP)

Genomic DNA was isolated from HeLa cells using DNeasy Blood & Tissue Kit (QIAGEN) in accordance with the manufacturer's protocol. Bisulfite conversion was performed in 1 μg of genomic DNA with an Epitect Bisulfite kit (QIAGEN) using the manufacturer's protocol. Converted DNA was amplified by heminested PCR and