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

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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

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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

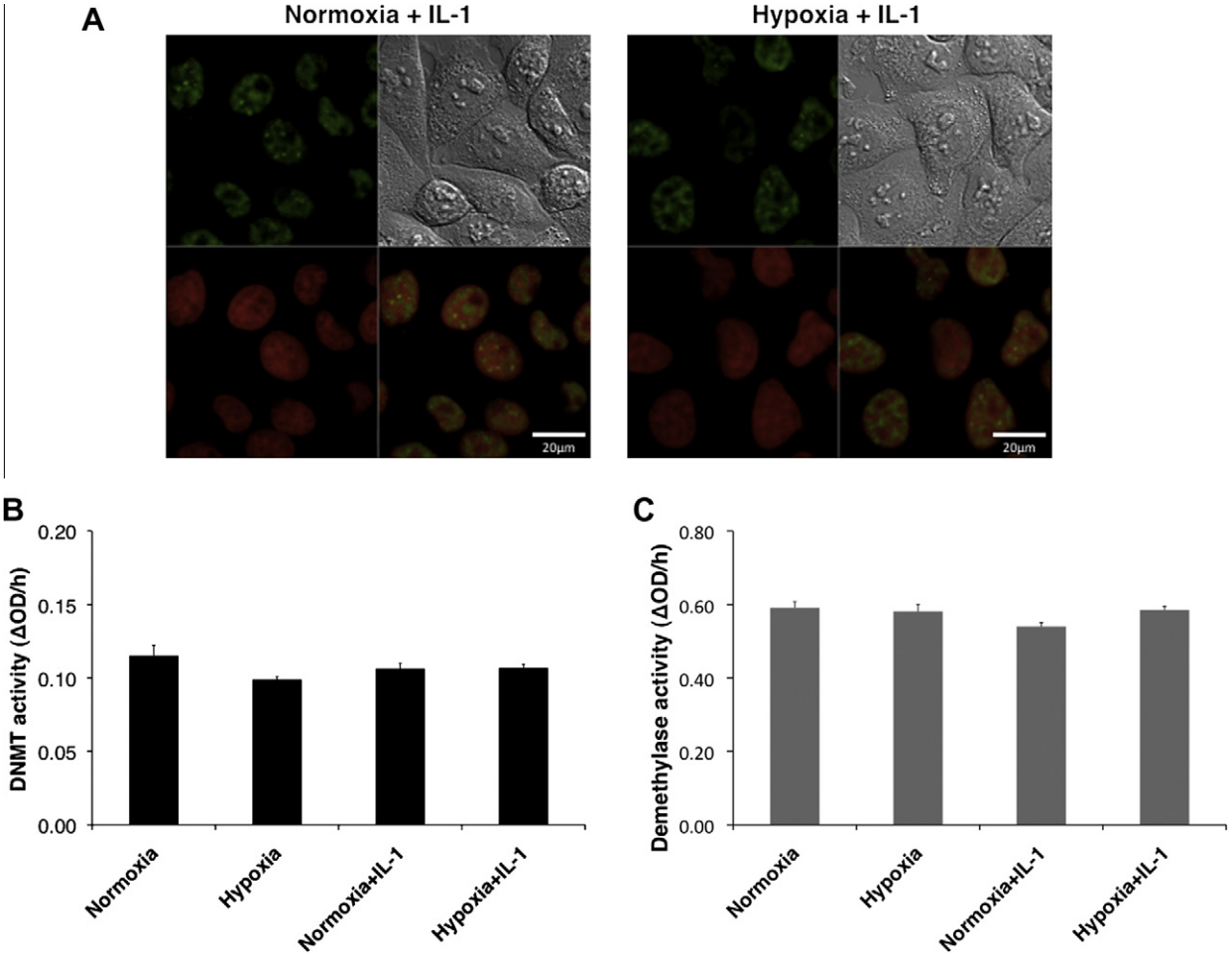

Fig. 1. Hypoxia does not alter the global epigenetic signature of DNA methylation. (A) Immunofluorescence staining of 5-methylcytosine (green) in HeLa cells treated with IL-1β under normoxic or hypoxic exposure for 6 h. Nuclei stained with propidium iodide shown in red. (B, C) DNMT activity (B) and DNA demethylase activity (C) in nuclear extracts of HeLa cells after exposure to hypoxia and/or IL-1β for 2 h. Data are representative of two independent experiments measured in hexaplicates.

cloned into a TOPO vector (Invitrogen). Primers targeting the MCP-1 5′-regulatory region (GenBank ID: D26087) were as follows:

MCP-1 enhancer,
Sense-1st, 5′-GGAGGAATGAAGAAAGTATTATTTATT-3′;
Sense-2nd, 5′-GGGTAATTGAGGATTTTGGATA-3′;
Antisense, 5′-ACCTCCCACTTCTACTCTATCAAA-3′;
MCP-1 promoter,
Sense, 5′-ATAGTTTATTTATTATTTATGGAAGATTTTTTTT-3′;
Antisense-1st, 5′-CCCTTATCCTTTTCTAAATCACCTTATCT-3′;
Antisense-2nd, 5′-AATCCATAATAACTCAAAAAAAAAACA-3′.

Vectors were sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). DNA methylation was analyzed using QUMA software (Quantification tool for Methylation Analysis; http://quma.cdb.riken.jp/).

2.8. Statistical analysis

All quantitative data in this study are represented as means ± the standard error of the mean (SEM) of three experiments, if not stated otherwise. Comparisons between groups were made using an unpaired two-tailed Student's *t*-test assuming equal variances. Differences were considered to be statistically significant at a level of $P < 0.05$.

3. Results

3.1. Hypoxia does not affect global DNA methylation

Hypoxic cells are characterized by global repression of RNA synthesis with simultaneous activation of selected genes [14]. Since transcriptional repression is commonly associated with DNA methylation [4], we examined the global profiles of DNA methylation in hypoxic cells. HeLa cells treated with IL-1β under normoxic or hypoxic conditions were stained with the antibody specific to 5-methylcytosine and assessed by immunocytochemistry. Our results showed that levels of global DNA methylation were not significantly different between cells treated with normoxia and hypoxia (Fig. 1A). Next, we examined the activity of DNMT and DNA demethylase in nuclear extracts from hypoxic cells. We could not observe significant changes in either DNMT (Fig. 1B) or DNA demethylase activities (Fig. 1C) after 2 h hypoxic exposure alone or in combination with IL-1β, consistent with our immunocytochemistry results.

3.2. Hypoxic repression of MCP-1 involves DNA methylation

Previously, we observed that hypoxia down-regulated MCP-1 mRNA and protein expression in human rheumatoid arthritis and osteoarthritis synovial fibroblasts, as well as in a HeLa cell line

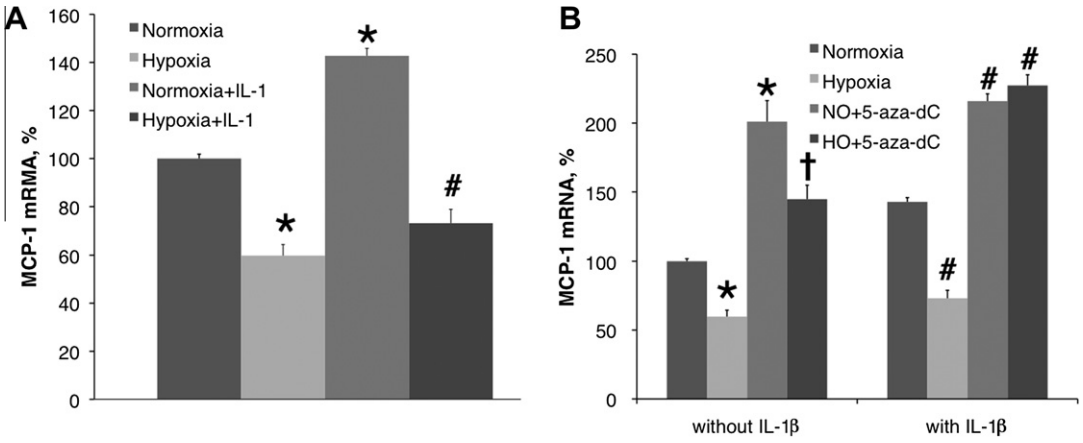

Fig. 2. Inhibitory effect of 5-Aza-2′-deoxycytidine (5-aza-dC) on hypoxia-induced repression of MCP-1 mRNA expression. (A) Effect of hypoxia on MCP-1 mRNA expression in cells treated with or without 50 ng/ml IL-1β for 6 h. Quantities of MCP-1 mRNA were normalized by the expression of 18S rRNA. The value of normoxic samples without IL-1β treatment was set as 100%. (B) Pretreatment with 5-aza-dC for 48 h abolishes the inhibitory effect of hypoxia on MCP-1 mRNA expression in IL-1β-treated cells. The expression level in normoxic cells was set as 100%. Data are representative of two independent experiments and shown as the mean ± SEM of triplicate measurements. $^{*}P < 0.05$, significantly different versus normoxia; $^{\#}P < 0.05$, significantly different versus normoxic IL-1β-treated samples; $^{\dagger}P < 0.05$, significantly different versus 5-aza-dC-treated normoxic samples.

under IL-1β stimulation [15,16]. Hypoxia significantly repressed MCP-1 mRNA expression in HeLa cells with or without IL-1β stimulation (Fig. 2A).

Next, to examine the involvement of DNA methylation in MCP-1 repression by hypoxia, we used 5-aza-dC. Treatment of HeLa cells with 5-aza-dC increased the basal level of MCP-1 mRNA expression, suggesting the possible background DNA methylation of MCP-1 gene in intact cells (Fig. 2B). Although the treatment with 5-aza-dC failed to abolish the repressive effect of hypoxia without IL-1β stimulation, the inhibition of DNA methylation completely abolished the repression of IL-1β-induced MCP-1 mRNA expression by hypoxia (Fig. 2B).

3.3. Hypoxia increases the level of DNA methylation in the MCP-1 5′-regulatory sequence

Analysis of the MCP-1 5′-regulatory sequence with a program to predict DNA methylation (methylator, http://bio.dfci.harvard.edu/Methylator/index.html) showed the presence of 33 CpG sites which were not grouped in the CpG island (Fig. 3A). Regulation of MCP-1 transcription mainly depends on two distinct areas of the 5′-regulatory region: a distant enhancer and promoter [17]. Predicted CpG sites were located at a higher density within these regions: 6 CpG sites in the enhancer region and 7 CpG sites in the promoter region. Therefore, we selected these CpG sites to perform a detailed study of DNA methylation.

An assay using BSP showed a low methylation level of the MCP-1 5′-regulatory sequence in unstimulated cells. Treatment with IL-1β for 6 h led to demethylation of CpG sites in the MCP-1 enhancer region (Fig. 3B). On the other hand, there were no differences in the methylation level between normoxia and IL-1β-treated cells within CpG sites located on the MCP-1 promoter (Fig. 3C). Following exposure to hypoxia with IL-1β, both the enhancer and promoter showed increased DNA methylation. However, hypoxia alone did not alter the methylation level in the MCP-1 5′-regulatory sequence.

To further confirm the role of DNA methylation in hypoxic repression of MCP-1 gene using additional method, we performed a methylated DNA Immunoprecipitation assay with an anti-5methylcytosine antibody and showed that DNA methylation of the CpG sites in the enhancer and promoter regions of the MCP-1 5′-regulatory sequence were induced by hypoxia treatment (Fig. 3D and E). The methylation on the MCP-1 enhancer region had a peak at 1 h after hypoxia treatment, and that on the promoter persisted after 2 h. These data are in agreement with those obtained from BSP.

3.4. Hypoxic response of the histone 3 methylation within the MCP-1 regulatory region

Next, we examined the involvement of histone methylation in the mechanisms of hypoxia-mediated transcriptional repression. ChIP assays were performed with antibodies specific to the active chromatin marker, histone H3 trimethylated at lysine 4 (H3K4me3) and the repressive chromatin marker, histone H3 dimethylated at lysine 9 (H3K9me2). IL-1β alone induced a level of H3K4me3 within the MCP-1 enhancer slightly at 15 min and to a stronger extent 1 and 2 h after the treatment (Fig. 4A). Interestingly, pick-like kinetics at 15 min and 60 min after stimulation were also observed for the acetylation of histone H3 and recruitment of the p65 subunit of NF-κB to the MCP-1 enhancer (unpublished observation). A slight increase in H3K4me3 was detected for the MCP-1 promoter region (Fig. 4B). Hypoxic exposure alone had no effect on repressive H3K9me3 modification; however, we observed a decline in H3K4me3 levels at the MCP-1 enhancer with two picks at 15 min and 60 min (Fig. 4). Simultaneous treatment with IL-1β and hypoxia caused a strong increase in the repressive H3K9me2 within both the MCP-1 enhancer and promoter regions (Fig. 4A and B, right panels). The levels of H3K4me3 on the MCP-1 promoter and enhancer were also elevated at the same time points as those of H3K9me3 of hypoxia with IL-1β exposure, although this elevation was to a lesser extent (Fig. 4A and B, left panels). These findings suggested that histone 3 methylation is involved in MCP-1 repression in hypoxic environment. Such modifications have greater impact in the background of IL-1β stimulation, i.e. inflammation.

4. Discussion

Epigenetic mechanisms control gene expression patterns without affecting the actual DNA sequence, and these patterns can be passed onto the daughter cells upon cell division. There are two molecular mechanisms that mediate epigenetic phenomena: DNA methylation and histone modifications. DNA methylation is thought to be the critical epigenetic modification linked to gene

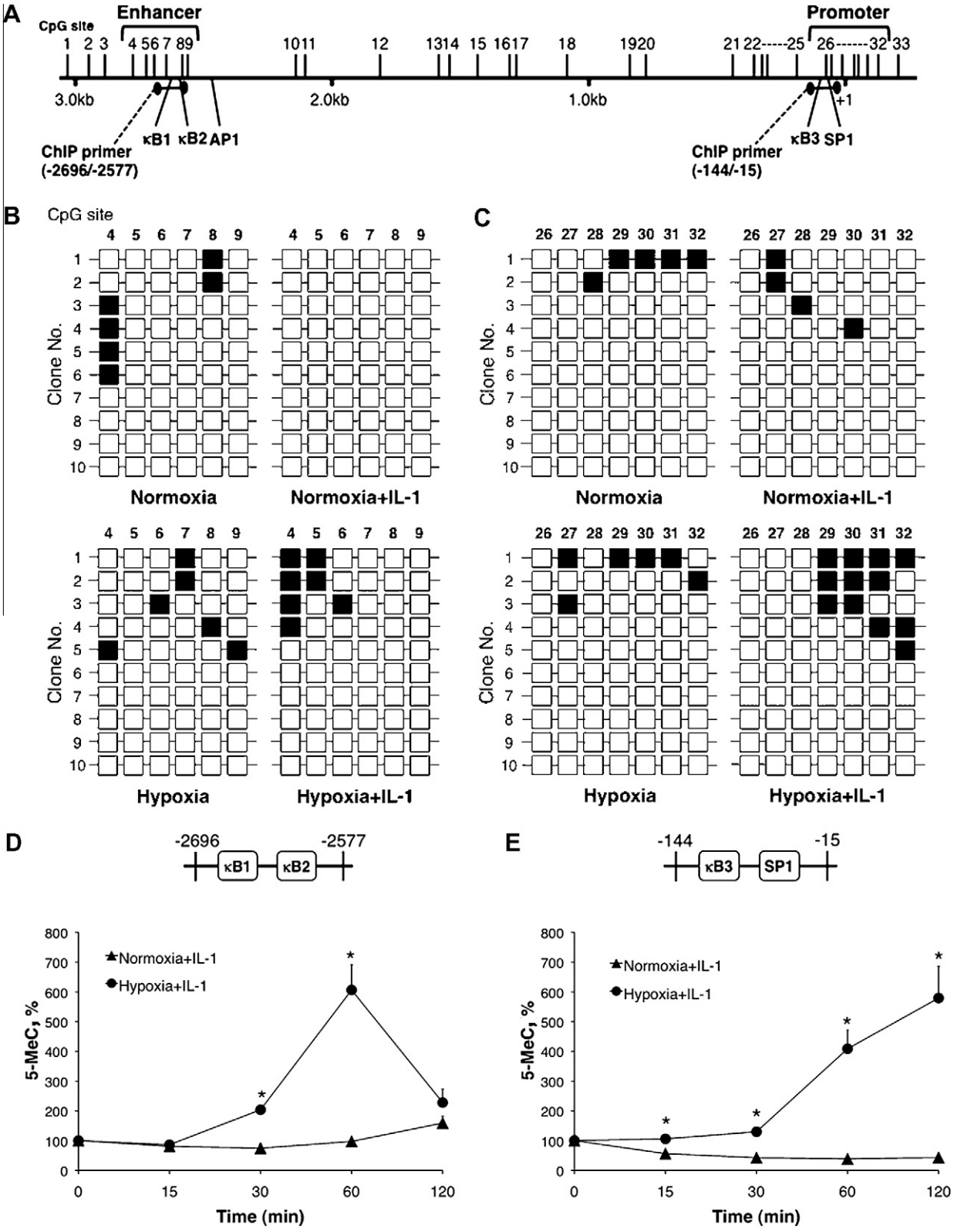

Fig. 3. Hypoxia increases the level of DNA methylation within the 5′-regulatory sequence of the MCP-1 gene in HeLa cells treated with IL-1β. (A) Structure of the MCP-1 gene regulatory region, representation of the predicted CpG sites and localization of the ChIP-qPCR primer sets. Individual CpG sites are indicated by numbers. (B, C) Effect of hypoxia on the methylation status of individual CpG sites in the MCP-1 enhancer region (B) and promoter region (C) analyzed by BSP. Open and filled squares indicate unmethylated and methylated CpGs, respectively. (D, E) Effect of hypoxia on the DNA methylation of the MCP-1 gene enhancer (D) and promoter (E) regions in HeLa cells treated with IL-1β determined by 5-methylcytosine immunoprecipitation. Data are expressed as a percentage of CpG methylation at 0 min. $^{*}P < 0.05$, significantly different versus normoxic IL-1β-treated samples.

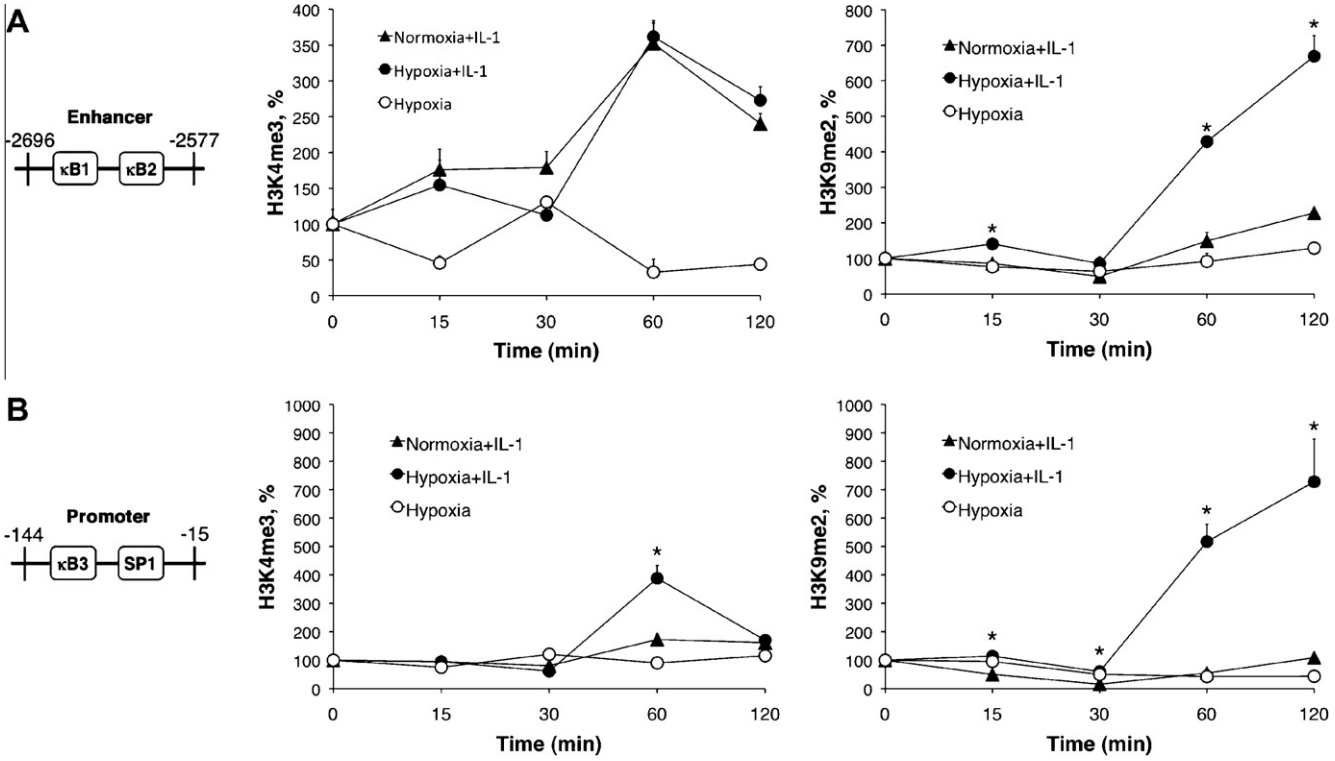

Fig. 4. Effect of IL-1β and hypoxia on the levels of histone 3 methylation within MCP-1 enhancer (A) and promoter (B) of MCP-1 gene regulatory region assessed by ChIP assays. Data are expressed as a percentage of histone methylation at 0 min. $^{*}P < 0.05$, significantly different versus normoxic IL-1β-treated samples.

repression, but methylation changes can also arise secondary to chromatin modifications and maintain boundaries between active and inactive chromatin [18]. Because epigenetic states are reversible they can be modified by environmental pressures, such as aging, diet and metabolic stress [19]. Epigenetic changes in cancer are linked to gene activation, gene silencing and chromosomal instability and are more clearly associated with tumor progression that are specific mutations [20]. Recent studies suggested that epigenetic reprogramming plays a role in the etiology of inflammatory diseases as well [21,22].

Hypoxic conditions can arise under the above mentioned pathological states, including inflammatory, fibrotic, and tumorigenic processes, and can also occur as a natural feature of aging organs. Although recent molecular findings linked inflammatory hypoxia to epigenome configurations, we observed that hypoxic treatment for 6 h did not change the level of global DNA methylation in HeLa cells. The data were supported by observations on unchanged cellular DNMT and DNA demethylase activities. Unlike relatively short hypoxia exposure, as in our study, chronic prolonged hypoxia affects global epigenetic programming. Significant increases in global levels of DNA methylation were observed in human prostate androgen-dependent cells which were permanently maintained under hypoxic conditions [23]. Exposure to severe hypoxic conditions of human colorectal and melanoma cancer cell lines induced losses of global methylation [24]. The same group has shown that although total activity levels of DNMTs were reduced in colorectal carcinoma cells exposed to hypoxia, bisulfite sequencing demonstrated a statistically significant increase in methylation of the $p16^{INK4a}$ promoter [25].

It is important to note that although the extensive methylation found in the bulk of chromatin of solid hypoxic tumors is reduced, the normally unmethylated CpG islands become hypermethylated [24]. Therefore, changes in DNA methylation within certain genes may appear more significant than the level of global DNA methylation. In our study, 5-aza-dC, an inhibitor of DNA methylation, completely abolished hypoxic repression of MCP-1 gene expression in IL-1β-treated cells. Consistent with this notion, we found that hypoxic treatment led to the increased methylation of CpG sites in the MCP-1 enhancer and promoter regions.

Two mechanisms of transcriptional repression resulting from DNA methylation are under consideration. One is the direct inhibition of transcription factor binding when methylation occurred in a DNA recognition element, the other is recruitment of histone deacetylases via methyl-CpG binding domain (MBD) proteins [26]. Both mechanisms contribute to tissue-specific silencing of the hypoxia-responsive human erythropoietin gene [27]. The high CpG content in some promoters of primary response genes by toll-like receptors can be responsible for low nucleosome occupancy and nucleosome instability, facilitating constitutive expression and rapid induction [28].

DNA methylation is functionally related to histone methylation in gene silencing. Treatment with 5-aza-dC, known to inhibit cytosine methylation, reduced the levels of di-methylated H3K9 and induced levels of di-methylated H3K4 at the $p14^{ARF}/p16^{INK4a}$ locus in cancer cells [29]. The inhibitory effect of 5-aza-dC on histone H3K4 methylation was observed in IL-1β-stimulated A549 cells, suggesting a role for histone methylation in acute inflammatory responses [30]. Hypoxia alters post-translational methylation of total histone populations, resulting in both activation and repression of gene transcription [14]. In our study, we observed that repressive H3K9me2 levels within both MCP-1 enhancer and promoter regions were strongly induced by co-treatment with IL-1β and hypoxia compared to IL-1β alone. Increased H3K9me2 levels in response to hypoxia were also demonstrated by another group with repression of neprilysin gene expression in primary cortical neurons [31].

We observed that hypoxia-mediated changes in the levels of H3K9me2 1 h after stimulation were accompanied by simulta-

neous increases in H3K4me3, although to a lesser extent. Interestingly, although tri-methylation of H3K4 residues is thought to be associated with actively transcribed genes, a recent study identified a class of genes that are characterized by H3K4me3 occupancy and transcription initiation, but show no evidence of elongation [32]. Therefore, such genes are predominantly regulated at post-initiation steps. Our unpublished observations show that this is the case for the MCP-1 gene, in which hypoxia represses productive elongation rather than recruitment of RNA Polymerase II.

In summary, our study demonstrated that hypoxia reduced MCP-1 expression, which is mediated by the increase in DNA methylation and repressive H3K9 di-methylation within the MCP-1 5′-regulatory region. In contrast to co-treatment with IL-1β and hypoxia, hypoxia alone did not strongly affect the methylation level of either DNA or histone. These results suggest that epigenetic regulation may be involved mainly in the hypoxic condition associated with inflammation. Our results support the novel thinking that DNA methylation is associated with not only permanent epigenetic gene silencing, but also with transient transcriptional repression, under environmental pressures. The concept of epigenetic regulation is gradually being recognized as an important factor in the pathogenesis of various diseases and may provide an excellent therapeutic target. Resent research provides an essential link between hypoxic inflammation and reprogramming of the epigenome [3]. However, the detailed molecular mechanism of DNA methylation-mediated hypoxic repression needs to be determined.

Acknowledgments

This work was supported by grants from the Japan Society for the Promotion of Science and from the Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases.

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