Lipopolysaccharide Induces Aberrant Hypermethylation of *Hic-1* in Mouse Embryonic Fibroblasts Lacking *p53* Gene

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Abstract. *Background: The present study asked whether continuous administration with lipopolysaccharide (LPS), a potent inflammatory agent, induces aberrant methylation in the promoter region of tumor suppressor genes and p53 and/or inducible nitric oxide synthase (iNOS) genes involved in its aberrant methylation. Materials and Methods: Mouse embryonic fibroblasts (MEFs) were prepared from mice harboring four different genotypes ($p53^{+/+}iNOS^{+/+}$, $p53^{+/+}iNOS^{-/-}$, $p53^{-/-}iNOS^{+/+}$ and $p53^{-/-}iNOS^{-/-}$). The MEFs were immortalized by 3T3 procedure and continuously cultured under a medium containing LPS or LPS plus interferon (IFN)-γ during 40 passages. The methylation status in the CpG site of hypermethylated in cancer-1 (Hic-1) exon 1a and p16 promoter region was monitored using bisulfite-sequencing methods. Results: LPS and LPS plus IFN-γ induced de novo methylation in the CpG sites of the Hic-1 gene. This site was methylated only in $p53^{-/-}$ MEFs, and the mRNA expression of Hic-1 decreased in $p53^{-/-}$ MEFs compared to $p53^{+/+}$ MEFs. The methylation patterns of Hic-1, however, were not affected by iNOS gene status. The promoter region of p16 was methylated by increasing the passage, even under the control medium, with LPS administration promoting methylation, particularly in MEFs lacking the iNOS gene. However, the methylation pattern was not significantly different between the p53 genotypes. Conclusion: Our preliminary study suggests that LPS induces de novo methylation in the CpG site in MEFs. For the Hic-1 gene, but not p16, the p53 gene might protect against aberrant methylation. The iNOS gene might not be involved in methylation of the Hic-1 gene, whereas the promoter region of p16 could be prone to methylation in MEFs lacking the iNOS gene.*

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It is widely accepted that multiple epigenetic alternations in tumor-suppressor genes and tumor-related genes can lead to carcinogenesis (1). In particular, the CpG island hypermethylation of the gene promoter regions is a frequent and early event in carcinogenesis.

Extended research has been conducted mainly in inflammation-associated cancers such as those of the stomach, liver and colon (2, 3). Genome-wide screening methods have shown that the CpG sites in the promoter region of many cancer-related genes are frequently methylated (4, 5). Furthermore, Stenvinkel *et al*. reported that global DNA hypermethylation was observed in the peripheral blood leukocytes of patients with inflammation (6). Such evidence suggests that chronic inflammation causes aberrant DNA hypermethylation. The epigenetic genes such as DNA methyltransferases (*Dnmt*), methyl-CpG-binding domain proteins, histone deacetylases, histone methyltransferases, and histone demethylases can be targeted by mutations and expression changes in the inflammatory process (7), but the molecular mechanisms remain poorly understood.

Several prominent electophilic mediators have been identified under conditions of chronic inflammation, among which a high amount of nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) and its metabolites are strongly suspected of affecting cancer promotion and progression *via* chronic inflammation (8). Hmadcha *et al*. (9) reported that nitric oxide (NO) transiently accelerated CpG island methylation in the promoter region of *IL-1 beta via* the posttranscriptional activation of DNA methyltransferase activity. In addition, NO exposure acts as a carcinogen by inducing the methylation of cytosine resulting in C-T transitions, and by enabling NO to silence the viral genome by inducing methylation (10, 11). The chronic effects of NO on methylation in the promoter regions of tumor suppressor genes, however, are largely unknown.

In carcinogenic processes, *iNOS* gene expression and the various biological effects of NO can be affected by *p53* status (12, 13). It was reported that NO-induced DNA damage and apoptosis depend on *p53* status (14, 15). We have shown the interactive effects of *p53* and *iNOS* gene

activity on gene expression such as cytokines (16). These findings indicated that *p53* status plays important roles in carcinogenic processes *via iNOS* gene activity. Moreover, p53 protein acts as a very important gatekeeper molecule through its regulation of DNA repair, the cell cycle and apoptosis to protect cells from chromosomal instability, and loss of p53 function increases susceptibility to malignant transformation [reviewed in (17)]. It is, therefore, of great interest whether *p53* might be associated with the aberrant methylation of tumor suppressor genes.

There are few *in vitro* models that enable methylation changes to be observed. The chief aim of this preliminary study was to examine: i) whether the continuous administration of lipopolysaccaride (LPS), a potent inflammatory agent, induces aberrant methylation in the promoter region of tumor suppressor genes, *hypermethylated in cancer-1* (*Hic-1*) and *p16*, in mouse embryonic fibroblasts (MEFs) and ii) whether *p53* and/or *iNOS* genes are involved in the methylation of CpG islands in the promoter region. We selected *Hic-1* and *p16* because it was reported that the functional loss of *Hic-1* and *p16* by hypermethylation is involved in the tumorigenesis of mouse fibrosarcoma (18, 19).

Materials and Methods

Mice and MEF isolations. Using mice harboring four different genotypes, $p53^{+/+}iNOS^{+/+}$, $p53^{-/-}iNOS^{+/+}$, $p53^{+/+}iNOS^{-/-}$ and $p53^{-/-}NOS^{-/-}$ (backcrossed to C57BL/6J) (16), mouse embryonic fibroblasts (MEFs) were prepared from 13.5-days-old postcoitus embryos as described elsewhere (20). Cells were grown for 2 days until the culture was 80-90% confluent, then harvested, viably frozen and labeled as passage P(0). For experiments using cells cultured in 20% oxygen/5% CO_2, MEFs were cultured at 37˚C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/ml glucose (Gibco), supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, HEPES (25 mM), 2-mercaptoethanol (0.1 mM) and antibiotic mix (Gibco). For immortalization, 3 million cells were trypsinized and replated in 10-cm culture dishes every 3 days (3T3 procedure). Immortalized cells with four genotypes each were obtained as a start passage S(0).

Experimental protocol. The cells having each genotype were cultured in a medium containing LPS (0, 0.1, or 1 μg/ml), or LPS (0, 0.1, or 1 μg/ml) (from *Escherichia coli*, 0111:B4; Sigma, St. Louis, USA) plus mouse interferon gamma (IFN-γ, 100 U/ml Wako Chemical Co. Ltd., Osaka, Japan) in a 6-well plate. Cells were grown until the culture was confluent and the passage was repeated using trypsin. These experiments were performed in a duplicate manner and continued to passages 40 [S(40)]. In the subsequent 10 passages [S(50)], the MEFs were cultured in a medium excluding LPS or LPS plus IFN-γ in order to examine whether the methylation status would change under such conditions.

Examination of p53 mutation. The mutation in *p53* gene from exon 1 to exon 8 was examined using the PCR-direct sequence method. Total RNA was converted into cDNA and amplified using the primer set of forward (F): 5'-GCATTCAGGCCCTCATCCTC-3' and reverse (R): 5'-GAGTGGAATCTGGGATTGTGTCT-3'. The PCR products were electrophoresed, and the band was cut-out. The purified PCR products were then sequenced using the reverse primers R1: 5'-CGCGGACACGGCTCCCAGCT-3', R2: 5'-TGGA GTCTTCCAGTGTGATG-3', R3: 5'-CGCGGATCTTGAGGGTGA AAT-3', and R4: 5'-CAGCAAGGAGAGGGGGAGGC-3'.

Analysis of NO production. A total of 0.5×10^5 cells of MEF S(0) and S(40) were distributed into 6-well plates and incubated in a medium of LPS alone (1 μg/ml) or LPS (1 μg/ml) plus IFN-γ (100 U/ml) for 5 days. NO_2 was measured by the Griess method using a commercial NO_2/NO_3 Assay kit (Dojindo, Kumamoto, Japan).

Bisulfite treatment. DNA was isolated from cells using a QIAamp DNA MiniKit (Qiagen, Maryland, USA). DNA (0.5 μg) was subjected to bisulfite treatment using an EZ DNA Methylation Kit™ (Zymo Research, Orange, USA). Bisulfite-treated DNA was stored at –20˚C until use.

Bisulfite DNA sequencing analysis. Bisulfite-treated DNA (1 μl) was amplified using a pair of specific primers in a total volume of 50 μl. For bisulfite genomic sequencing of mouse *Hic-1*, exon 1a was amplified using primer pairs: *Hic-1a* forward: 5'-GGGTTGTT TTAGATAAGAGTGTG-3', reverse: 5'-AAAAACACTTAAAACC CCAA-3' (18).

To determine methylation in the promoter region of *p16*, the bisulfite-modified DNA was amplified in nested PCR amplification reactions with specific primers as described elsewhere (21). Briefly, the outer reaction was performed using primers FM1 (5'-GTTGT GTATAGAATTTTAGTATTG-3') and RM2 (5'-CCACCCTAACC AAT CTATCTA CAAC-3') for 25 cycles (denaturation at 94˚C for 1 min, annealing at 52˚C for 1 min, extension at 72˚C for 1 min). A 1:10 dilution was made from the outer reaction and 2 μl was used as the template in a 50 μl inner reaction (30 cycles of denaturation at 94˚C for 1 min, annealing at 56˚C (set 1) or 53˚C (set 2) for 1 min, 72˚C for 1 min and 7 min/cycle extension). The inner PCR reaction was performed using two different primer sets: set 1: FM3 (5'-TTTTAATATTTGGGTGTTGTATTG-3'), RM4 (5'-ACCCAAAC TAC AAA AAA AAT ACA-3'); set 2: 20FM (5'-GGTGTTTA ATTTATGTTATATTTA-3') and RM2.

PCR products were electrophoresed on 2% agarose gel and visualized using ethidium bromide. PCR products were directly purified using the Qiagen Kit. PCR products were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) and primers: *Hic-1*:5'-CCAAA CACAACCCAAACACCC-3 and *p16*:5'-CCCAAACTACAAAA AAAATACAAAAT-3' and RM2.

RT-PCR. MEFs were harvested when they reached 100% confluence. Total RNA was isolated from cells using a RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized with a commercial kit (ReverTra Ace qPCR RT kit; Toyobo Co., Ltd., Osaka, Japan) in a reaction volume of 15 μl containing 5 μg of total RNA and 0.2 μg of random hexamer primers, according to the manufacturer's instructions. To detect the following genes, the first-strand cDNA was amplified by PCR with the following sets of oligonucleotide primers: *Hic-1* F: 5'-CCTGCGACAAGAGCTACAAGGAC-3', R: 5'-GAGGCTCTCCA GCGCCACCTTG-3'; *p16* F: 5'-AGTCCGCTGCAGACAGACTG-3', R: 5'-CGGGAGAAGGTAGTGGGGTC-3'; *Dnmt1* F: 5'-CCCATG CATAGGTTCACTTCCTTC-3', R: 5'-TGGCTTCGTCGTAACTCT

CTACCT-3'; *Dnmt3a* F: 5'-GCACCTATGGGCTGCTGCGAAGA CG-3', R: 5'-CTGCCTCCAATCACCAGGTCGAATG-3'; *Dnmt3b* F: 5'-CAAGGAGGGCGACAACCGTCCATT-3', R: 5'-TGTTGGACAC GTCCGTGTAGTGAG-3'.

Samples were amplified in 25 cycles for *β-actin*, 30 cycles for *Dnmt1, Dnmt3a, Dnmt3b* and *p16*, and 35 cycles for *Hic-1* (denaturation at 94˚C for 30 s, annealing at 55˚C for 60 s and extension at 72˚C for 120 s). The PCR products were visualized after electrophoresis on 2% agarose gels by staining with ethidium bromide.

Results

First, we determined whether any mutations were present in the *p53* gene by PCR-direct sequencing in the region that covered all of the whole exons because once MEFs harboring the *p53* wild-type were immortalized by the 3T3 procedure, the normal room O_2 level could cause some mutations in the *p53* gene during passages (22). In this study, no mutation was found in $p53^{+/+}$ MEF S(40).

iNOS induction and p53 status. In this study, iNOS was induced at a high and low level by LPS plus IFN-γ and LPS alone, respectively. Time course changes of mRNA expression were examined by RT-PCR. In $p53^{-/-}$ $iNOS^{+/+}$ MEF S(0), LPS (1 μg/ml) plus IFN-γ (100 U/ml) administration induced a marked induction of iNOS mRNA at a peak of 2 days after treatment (Figure 1A), with the concentration of nitrite reaching 30 μM in the medium at 5 days (Figure 1B). Both mRNA and NO_2 production were significantly greater in $p53^{-/-}$ $iNOS^{+/+}$ MEFs compared to that in $p53^{+/+}$ $iNOS^{+/+}$ MEFs (Figure 1B). NO_2 production by stimulation with LPS (1 μg/ml) plus IFN-γ (100 U/ml) was not demonstrably changed between $p53^{-/-}iNOS^{+/+}$ MEF S(0) and S(40) (29.9±12.0 μM), but decreased in $p53^{+/+}iNOS^{+/+}$ MEF S(40) (3.0±2.5 μM). These findings are consistent with previous results (12). Messenger RNA of *iNOS* was up-regulated for 5 days at a peak of 3 to 4 days after LPS alone (data not shown), with the maximum concentration being less than 5 μM in both $p53^{+/+}iNOS^{+/+}$ and $p53^{-/-}iNOS^{+/+}$ MEFs (Figure 1B).

Methylation status of Hic-1 exon 1a and p16 promoter regions and mRNA expression. We identified methylated cytosine residue in *Hic-1* exon 1a (1266-1579) by the bisulfite-sequence method. The original method reported by Chen *et al*. (18) was able to detect 33 CpG sites, whereas we could identify only 28. Five sites of the 5' portion were not clearly identified. Initially there was no methylation of *Hic-1* exon 1a in the 4 genotypes of MEFs; methylation of CpG increased once the number of passages exceeded 30 (data not shown). Figure 2A shows the methylation status of the *Hic-1* exon 1a region in MEF S(40) with each *p53* and *iNOS* genotype under the administration with LPS alone or LPS plus IFN-γ. In the control, there were no methylated sites of CpG in MEFs with the $p53^{-/-}iNOS^{+/+}$ genotype. LPS alone or LPS plus IFN-γ induced a significant methylation of CpG sites only in MEFs lacking the *p53* gene. Administration with LPS alone induced methylation in $p53^{-/-}iNOS^{+/+}$ MEFs. Conversely, administration with LPS plus IFN-γ induced more hypermethylation in $p53^{-/-}iNOS^{-/-}$ MEFs compared to that in $p53^{-/-}iNOS^{+/+}$.

Methylation status of the promoter region in *p16* was also examined in sites -1 to -9 (nucleotides -23 to -172) and -12 to -19 (nucleotides -307 to -433) (Figure 2B). In the control MEFs, methylated sites were found only in MEFs lacking the *iNOS* gene. LPS alone and LPS plus IFN-γ administration also induced methylation more frequently in $iNOS^{-/-}$ MEFs than in $iNOS^{+/+}$ MEFs. There was, however, no significant difference in methylation status between MEFs with and those without the p53 gene.

We examined the methylation status of *Hic-1* exon 1a in $p53^{-/-}$ MEF S(50) after the 10 passages without LPS or LPS plus IFN-γ in the culture medium in order to determine whether or not methylation would change. Figure 3 shows that the number of methylated CpGs increased, compared to that in MEF S(40) (Figure 2A), indicating that it is not a transient phenomenon and that the methylation of CpG sites has progressed without LPS or LPS plus IFN-γ.

We investigated the association between methylation patterns and gene expression (Figure 4). Expression of *Hic-1* mRNA was lower in $p53^{-/-}$ MEFs compared to $p53^{+/+}$ MEFs. On the other hand, *p16* mRNA expression was lower in $iNOS^{-/-}$ MEFs compared to that in $iNOS^{+/+}$ MEFs. These expression changes were consistent with the methylation patterns (Figure 2).

Dnmt expression. Messenger RNA expression of *Dnmt1, Dnmt3a* and *Dnmt3b* were examined by RT-PCR in MEF S(40). Expression of *Dnmt1* was higher in $p53^{-/-}$ compared to $p53^{+/+}$ MEFs. LPS or LPS plus IFN-γ treatment increased the expression of *Dnmt1, Dnmt3a* and *Dnmt3b* in $p53^{-/-}iNOS^{+/+}$ MEFs, while LPS administration enhanced the expression of *Dnmt3a* and *3b* in $p53^{-/-}iNOS^{-/-}$ MEFs (Figure 5).

Discussion

This preliminary study showed that LPS, a potent inflammatory agent, induced *de novo* methylation in the CpG site of the promoter region of *Hic-1*, a tumor suppressor gene, and that the *p53* status possibly affected the methylation.

Hic-1 is hypermethylated and transcriptionally silent in some types of human cancer (23). Mice suffering heterozygous disruption of the *Hic-1* gene develop squamous cell carcinoma, lymphoma and sarcoma. The region exon 1a that we examined was methylated mainly in sarcoma, but not in the carcinoma

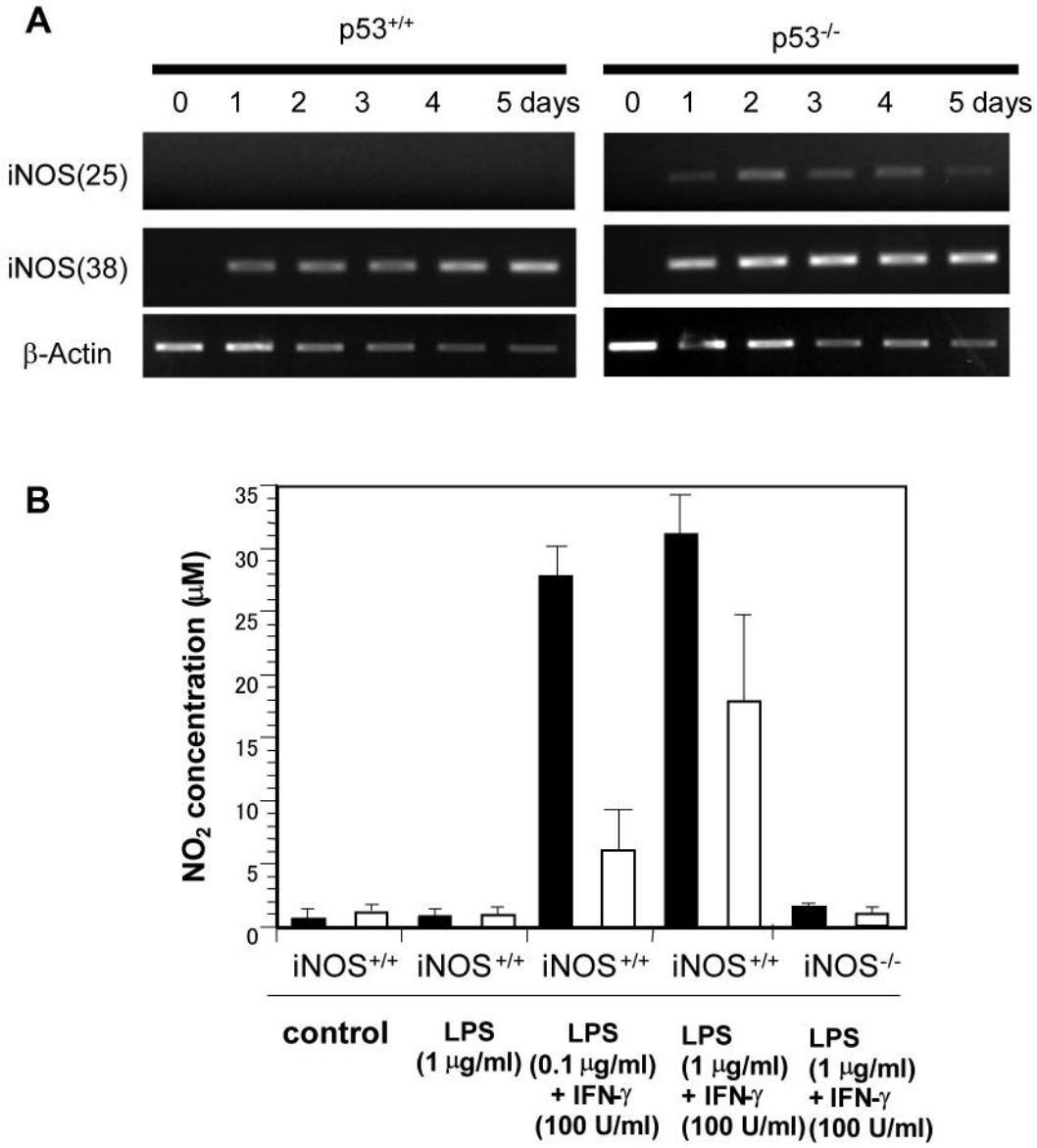

Figure 1. *Expression of iNOS mRNA and NO production after administration of LPS plus IFN-γ. A) Time course changes of iNOS mRNA were examined in p53$^{+/+}$ and p53$^{-/-}$ MEFs after stimulation with LPS (1 μg/ml) plus IFN-γ (100 U/ml). The numbers 25 and 38 indicate PCR cycles. B) Nitrite concentration was measured in the medium 5 days after stimulation by the Griess method. Shaded and open bars indicate mean (±SD) in p53$^{-/-}$ and p53$^{+/+}$ MEF S(0), respectively.*

that developed in *Hic-1*$^{+/-}$ mice. In addition, *Hic-1* and *p53* functionally cooperate in tumor suppression, and a functional loss of the *Hic-1* gene accelerates tumorigenesis in *p53*$^{+/-}$ mice (18). It has been reported that the promoter region of *Hic-1* has a sequence that can be associated with p53, and that p53 transcriptionally regulates *Hic-1* gene expression (24). Our study found aberrant methylation in *p53*$^{-/-}$ MEFs, but not in *p53*$^{+/+}$ MEFs by continuous administration with LPS. Thus we compared the expression level of *Hic-1* mRNA with the status of *p53* gene: the expression of *Hic-1* mRNA was lower in *p53*$^{-/-}$ MEFs compared to *p53*$^{+/+}$ MEFs. These results suggest that *p53* might protect the methylation of *Hic-1,* which would be consistent with previous findings by Chen *et al.* that hypermethylation of *Hic-1* is frequent only in tumors with *p53* mutations (18).

In cancer cases, CpG sites in the promoter are widely and completely methylated (dense methylation) when a gene is silenced by methylation of the promoter. Aging, chronic inflammation and viral infection are involved in the induction of 'seeds of methylation', which are an important precursor (1). In this study, we examined the changes in the methylation pattern of *Hic-1* after 10 passages in the control

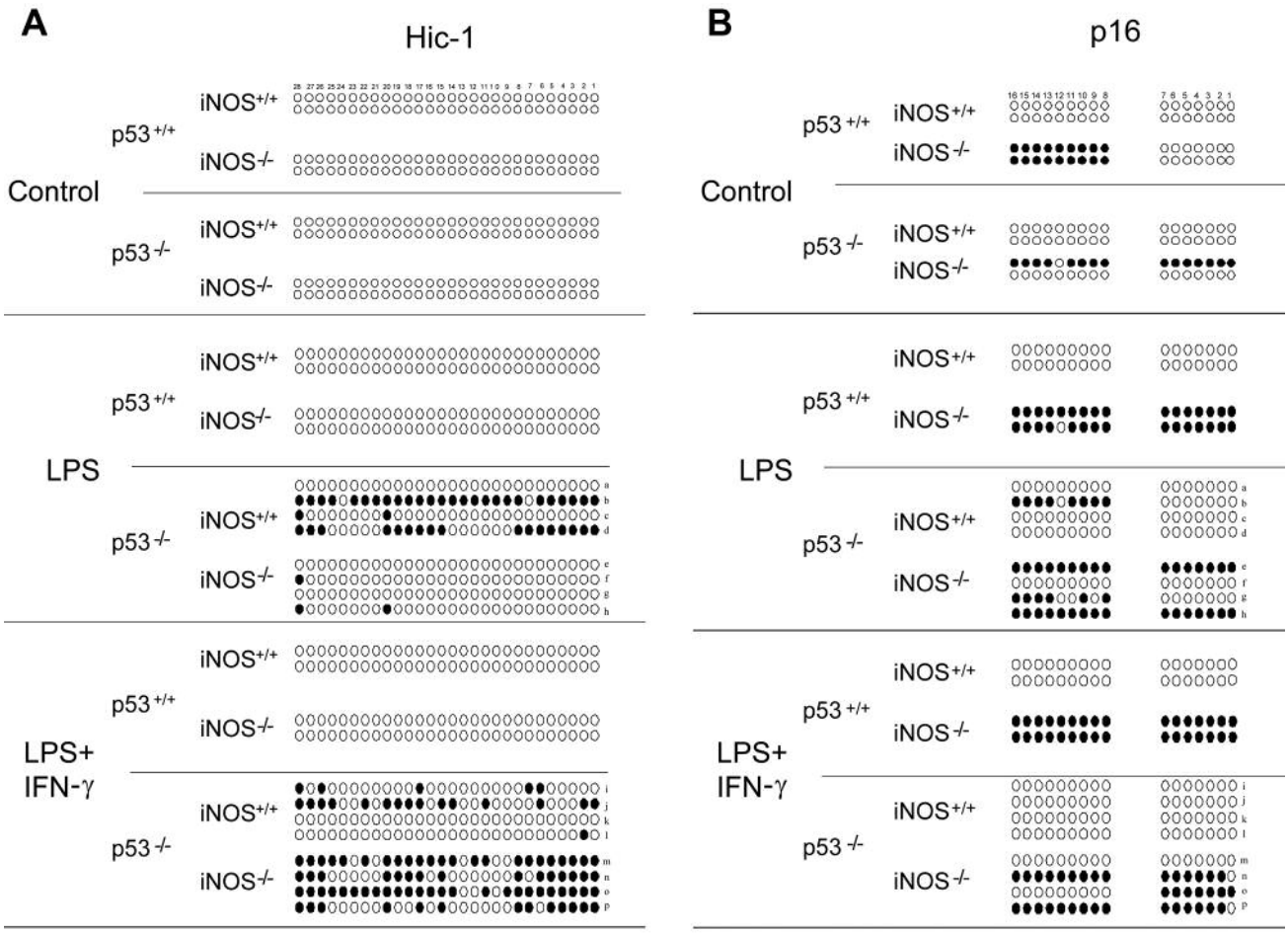

Figure 2. *Status of methylation of Hic-1 and p16 in MEF S(40). In MEF S(40), open and closed circles denote unmethylated and methylated CpG sites, respectively. A) 28 CpG sites (1266-1579) of Hic-1 exon 1a (GenBank mouse Hic1 genomic sequence AF036582). B) 16 CpG sites of p16 (No. 1 to 7 and 8 to 16 correspond to nucleotides –23 to –172 and –307 to –433 in GenBank accession no. U47018). Letters ('a' to 'p') correspond to MEF S(50) in Figure 3. One μg/ml of LPS was administered, except for MEFs of a, b, e, f, i, j, m and n (LPS: 0.1 μg/ml).*

medium, showing that methylation progressed when the seeds of methylation were frequently monitored. This suggests that inflammation can induce the seeds of methylation in the CpG site and, once the methylation progresses, the methylated CpG could be irreversible and lead to dense methylation even if the stimulation by inflammation has ceased.

The methylation of CpG sites of the *p16* promoter region examined in this study appears to play an important role in the transcriptional repression of *p16* (25). Evidence from several studies confirms the involvement of a *p16* aberration, particularly in inflammation-associated cancer such as gastric cancer and hepatocellular carcinomas (26, 27). Animal experiments also showed that oxidative stress could cause the hypermethylation of *p16* promoter region in rat renal cell carcinoma (28), nickel induced fibrosarcoma in $p53^{+/-}$ mice (19), and liver tissue after chronic low-dose X-

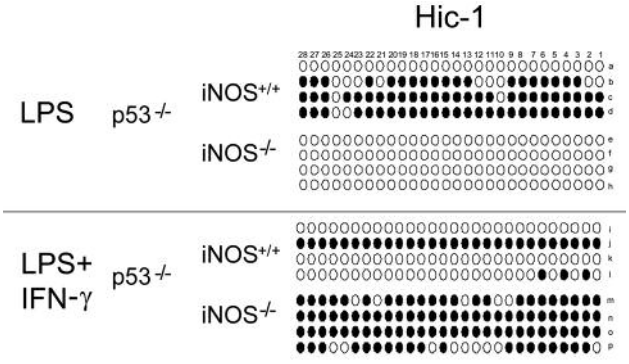

Figure 3. *Status of methylation of Hic-1 in MEF S(50). Changes in methylated CpG were examined in $p53^{-/-}$ MEF S(50); 10 passages for MEF S(40)were performed in medium lacking both LPS and LPS+IFN-γ. Unmethylated and methylated CpG sites are denoted by open and closed circles, respectively. Letters ('a' to 'p') correspond to MEF S(40) in Figure 2.*

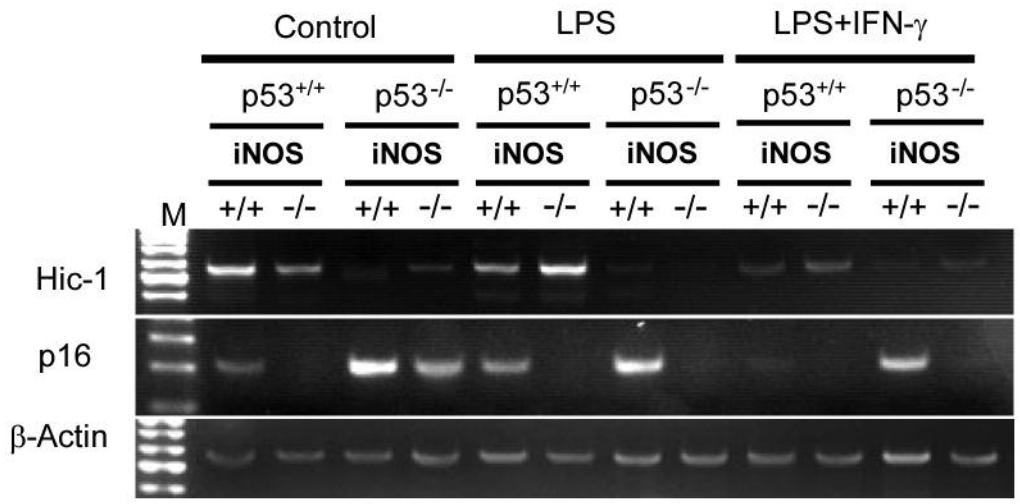

Figure 4. *mRNA expression of Hic-1 and p16. mRNA expression levels of Hic-1 and p16 in MEF S(40) were examined by RT-PCR.*

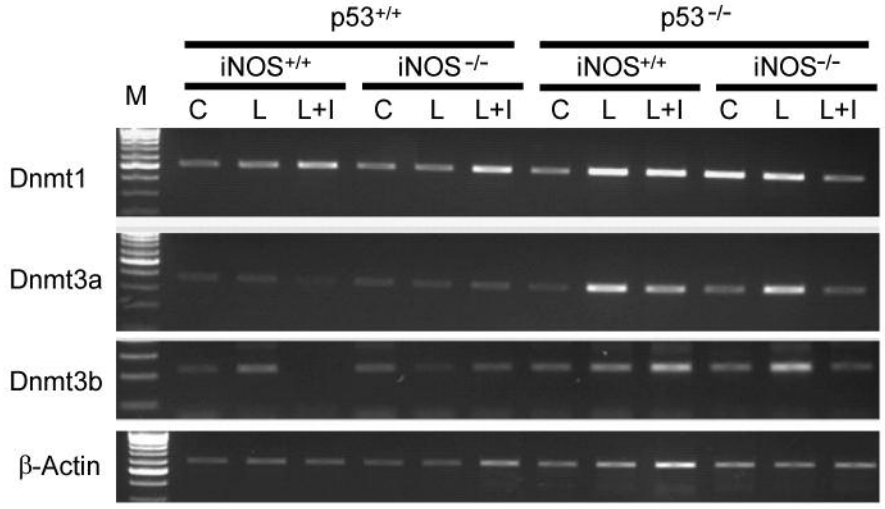

Figure 5. *mRNA expression of Dnmt1, Dnmt3a and Dnmt3b. The levels of these three mRNA expressions in MEF S(40) were examined by RT-PCR. M: marker, C: control, L: LPS, L+I: LPS + IFN-γ.*

ray irradiation (29). Recently Kostka *et al.* (30) have reported that phenobarbital, a non-genotoxic carcinogen and iNOS inducer, caused a transient change in the methylation status of the *p16* promoter region. Thus, we examined the role of the *iNOS* gene in methylation of the *p16* promoter region. In the present study, however, we frequently observed hypermethylation of the *p16* promoter regions in MEFs lacking the *iNOS* gene, suggesting that NO might protect that region from methylation.

Methylation of DNA is associated with cell aging (31). LPS promotes signaling in the cell cycle. It is speculated that the cell proliferation was accelerated by administration with LPS and that it induced *de novo* methylation. Thus the number of cell divisions was higher in MEFs cultured with LPS or LPS plus IFN-γ than that in the controls. However, we did not observe any increase in methylation after more than 20 passages: [S(60)] were performed under the control medium (unpublished data). The administration with LPS activates NF-κB signal transduction *via* toll-like receptors, enables mimicking of the inflammation process, and produces some oxidants such as superoxide and NO (32). Damaged cytosine products arising during the inflammatory process, particularly halogenated cytosine residues, would be expected to facilitate binding with methyl-binding protein and enzymatic methylation (33). We tested the effect of the addition of IFN-γ to LPS on the

methylation, since it is thought that IFN-γ can dramatically activate NF-κB *via* an up-regulation of the expression of toll-like receptors and can thus produce several kinds of oxidants (34). Our results showed that an increase in the number of methylated CpGs in both *Hic-1* and *p16* occurred with the administration of LPS plus IFN-γ compared to that of LPS alone. In addition, LPS plus IFN-γ administration significantly induced iNOS in $p53^{-/-}$ MEFs compared to $p53^{+/+}$ MEFs. Unexpectedly, the administration of LPS plus IFN-γ induced methylation in $p53^{-/-}iNOS^{-/-}$ MEFs. These findings suggest that oxygen-mediated cytosine damage might be involved in epigenetic alterations, but that *iNOS* gene status is not associated with the methylation of cytosine.

In this study, expressions of the DNA methyltransferases, *Dnmt3a* and *Dnmt3b*, were increased by long-term administration of LPS and of LPS plus IFN-γ, particularly in $p53^{-/-}$ MEFs. The methylation pattern of *p16*, however, was not associated with *p53* status. We also examined the CpG cluster of the *p21* promoter region (from –775 to –676) where these CpG sites were frequently methylated in tumors of $APC^{+/-}$ $p21^{+/-}$ mice (35). All CpG sites were methylated in MEFs with all genotypes and treatments (unpublished data). These results show that the mechanism was not attributed simply to the increased expression of DNA methyltranseferases. The mechanism of methylation involved might differ among target genes. Further extensive studies are needed to settle this matter.

In the carcinogenic process, hypomethylation of the genome and hypermethylation in the promoter region of tumor suppressor genes is a complex process. The mechanisms responsible for DNA methylation largely remain to be elucidated. Our study demonstrated the possible involvement of the *p53* and/or the *iNOS* gene in the methylation of tumor suppressor genes by chronic inflammation.

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