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Expression of heme oxygenase-1 in human leukemic cells and its regulation by transcriptional repressor Bach1

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Heme oxygenase (HO)-1 has anti-oxidative, anti-inflammatory, and anti-apoptotic activities. However, little is known about the regulation of HO-1 in human primary acute myeloid leukemia (AML) cells. Here we investigated the expression of HO-1 in primary and established AML cells as well as other types of leukemic cells and normal monocytes, and its regulatory mechanism by the transcriptional repressor, BTB and CNC homology 1 (Bach1), and the activator, nuclear factor erythroid-derived 2 related factor 2 (Nrf2). Leukemic cell lines such as U937 expressed little HO-1, whereas most freshly isolated AML cells and monocytes expressed substantial amounts of HO-1, along with Bach1 and Nrf2. When U937 cells were treated with phorbol myristate acetate (PHA) or γ-interferon, they significantly expressed both HO-1 and Bach1, like primary AML cells. Treatment with lipopolysaccharide (LPS) enhanced HO-1 expression in U937 cells but suppressed it in primary monocytes and PMA-treated U937 cells. In HO-1-expressing cells, Bach1 was localized in the cytoplasm, but Nrf2 was localized in the nuclei. Chromatin immunoprecipitation assay of these cells revealed the preferential binding of Nrf2 over Bach1 to Maf-recognition elements, the enhancer regions of the *HO-1* gene. The downregulation of the *HO-1* gene with siRNA increased a cytotoxic effect of an anticancer drug on primary AML cells, whereas the downregulation of Bach1 increased *HO-1* expression, leading to enhanced survival. These and other results show that Bach1 plays a critical role in regulating *HO-1* gene expression in AML cells and its expression suppresses their survival by downregulating *HO-1* expression. Thus, functional upregulation of Bach1 is a potential strategy for antileukemic therapy. (*Cancer Sci* 2010; 101: 1409–1416)

Heme oxygenase (HO)-1 is an inducible form of HO, which degrades heme into carbon monoxide, Fe^{2+}, and biliverdin. HO-1 possesses cytoprotective properties such as anti-oxidative, anti-inflammatory, and anti-apoptotic functions, and these properties are beneficial to cells, tissues, organs, and organisms.[1,2] Accumulating evidence shows that induction of HO-1 is a promising strategy for the treatment of various ischemic and inflammatory diseases.[1–3] In addition, HO-1 is a potential target for cancer therapy because this enzyme gives survival and growth advantages to malignant cells by means of its anti-apoptotic activity.[4–8] Aberrant expression of HO-1 in human cancers, including hematological malignancies, is implicated in oncogenesis and chemoresistance. In primary CML cells and the CML-derived K562 cell line, constitutively expressed HO-1 has been identified as a BCR/ABL oncoprotein-dependent survival factor.[9] Other studies have shown that modulation of HO-1 expression affects cellular growth or survival of myeloid leukemia cells.[10–12] Therefore, understanding the regulatory system for the HO-1 expression in myeloid lineage cells seems to be critically important.

HO-1 is induced in response to oxidative stress, and its expression is regulated by the binding of a transcription factor, nuclear factor erythroid-derived 2 related factor 2 (Nrf2), to Maf-recognition elements (MARE).[4] Upstream of the HO-1 coding region, EN1 and EN2 have been identified as enhancer regions containing MARE, where the transactivator Nrf2 and a transcriptional repressor, BTB and CNC homology 1 (Bach1), regulate *HO-1* gene expression positively and negatively, respectively, by forming heterodimers with the small-Maf protein.[13–18]

Nrf2 plays an essential role in *HO-1* gene regulation in human monocytes.[19,20] Tumor necrosis factor (TNF)-dependent HO-1 induction is mediated by the activation of Nrf2 in human myelomonocytic leukemic cell lines.[11] In contrast, we have previously shown that TNF suppresses HO-1 expression in peripheral monocytes by accelerating mRNA decay without affecting transcription activity.[21] Moreover, LPS positively or negatively regulates HO-1 expression in human and rodent monocytes and the human myelomonocytic leukemic cell line, THP-1.[19,22–24] Bach1 is known to play an important role in cellular homeostasis.[25] As the affinity of Bach1 for MARE is higher than that of any activator, Bach1 occupies the element and shuts off HO-1 expression under normal conditions.[14,15,26,27] When Bach1 is exported from the nuclei upon exposure to oxidative stresses, MARE becomes accessible to Nrf2, which leads to expression of HO-1.[28,29] Bach1-deficient mice, in which HO-1 expression is increased in myocardial and smooth muscle cells, are resistant to ischemic and pro-atherosclerotic stresses.[30,31]

Despite these previous findings, there is little information about HO-1 expression in leukemic cells from AML patients. In this study, we investigated the expression of HO-1 and its regulatory mechanisms by Nrf2 and Bach1 in human AML cells as well as mature monocytes and leukemic cell lines.

Materials and Methods

Patients and cell preparation. Primary leukemic cells were obtained from *de novo* AML and acute lymphoblastic leukemia (ALL) patients at diagnosis, before any therapy. AML cells were classified according to morphological and cytochemical findings based on French-American-British criteria.[32] Three subtypes of AML cells were categorized based on the stage at which normal differentiation was blocked in the leukemic blasts: M1, myeloblastic without maturation; M2, myeloblastic with maturation; and M4, myelomonocytic. Clinical profiles of the individual patients are shown in Table S1. Leukemic cells occupied more than 60% in whole circulating white blood cells in individual patients, as indicated by morphological analysis. All

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experiments were approved by the Ethics Committee of Yokohama City University Graduate School of Medicine (Yokohama, Japan) and informed consent was obtained in accordance with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation over Ficoll–Hypaque (ICN Biochemicals, Aurora, OH, USA). The purity of the leukemic cell population was constantly more than 80% in isolated PBMC (Table S1). $CD14^+$ monocytes were isolated from PBMC in healthy donors using MACS magnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) with human CD14 MicroBeads (Miltenyi Biotec) as previously described.[21]

Materials. The following materials were used: PMA and actinomycin D (Act D) from Sigma-Aldrich (Saint Louis, MO, USA); LPS (*Escherichia coli* O111: B4) from Calbiochem (La Jolla, CA, USA); recombinant human TNF-α and γ-interferon (IFN-γ) from R&D Systems (Minneapolis, MN, USA); recombinant human interleukin (IL)-18 from MBL (Nagoya, Japan); and antihuman Nrf2 (H-300) and (C-20), Bach1 (C-20), and Lamin A/C Abs from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The human Bach1 cDNA-encoding $pcDNA3.1^+$ was kindly provided by Dr. Tsutomu Toki (Hirosaki University Graduate School of Medicine, Hirosaki, Japan).[33] $pcDNA3.1^+$ (Invitrogen, Carlsbad, CA, USA) empty vector was used for the control.

Cell culture and stimulation protocol. U937, HL60, and K562 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). These cell lines, primary leukemic cells, and monocytes were maintained in RPMI-1640 supplemented with 10% FCS (Equitech-Bio, Kerrville, TX, USA), HEPES, L-glutamine, and antibiotics under 5% CO_2 in air at 37°C. In some experiments, cells were treated with 100 nM PMA for 48 h, further incubated in the fresh maintenance medium for an additional 24 h, and used as PMA-treated cells.[34] For the cell viability assay, siRNA-treated AML cells (5×10^5 per well) were incubated with 10 μM cytosine arabinoside (Ara-C; Sigma-Aldrich) in 96-well plates for 24 h, and assayed with a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Absorbance at 450 nm was measured for each culture. The sensitivity of AML cells to Ara-C was represented by a gap between the survival ratio of untreated AML cells and that of Ara-C-treated cells after 24 h.

Immunoblotting analysis. Protein expression levels were determined by SDS-PAGE and subsequent immunoblotting as previously described.[21] For several experiments, cytoplasmic and nuclear extracts were prepared from cells with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA).

Real-time PCR. Total RNA was extracted and cDNA was synthesized as described previously.[35] HO-1 transcript levels were quantified after PCR amplification as previously indicated.[21] The $\Delta\Delta C_T$ method was used to semiquantify mRNA levels, according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA).

Transfections with siRNA and plasmids. Human HO-1 siRNA and non-targeting negative control RNA were purchased from Ambion (Austin, TX, USA).[11] Human Bach1 siRNA and control RNA were obtained from Santa Cruz Biotechnology. Each siRNA was introduced into cells using Nucleofector II and Nucleofector Kit C (Amaxa Biosystems, Gaithersburg, MD, USA), and expression vectors were transfected using Nucleofector Kit V according to the manufacturer's protocol.[36,37] The transfection efficacy, which was evaluated by means of GFP-expressing plasmid pMAX-GFP (Amaxa Biosystems), was more than 40% in U937 cells (data not shown).

mRNA stability assay. PMA-treated U937 cells were incubated with or without Act D (5 μg/mL) for 1 h. Then the cells were stimulated with LPS (1 μg/mL) for a further 2.5 h. HO-1 mRNA levels were determined by real-time PCR.

Reporter gene assay. pHO-1(-4.5k) and pHO-1(-4.0k) were used, both of which were constructed by the human HO-1 promoter region-encoding firefly luciferase plasmid pGL3 HO-1.[21] The MARE EN1 region was contained in pHO-1(-4.5k) but not in pHO-1(-4.0k). U937 cells (1×10^6 per well) were treated with PMA (100 nM) for 24 h in 24-well plates, transfected with 2 μg pHO-1(-4.5k), pHO-1(-4.0k), or mock pGL3 basic vector in the presence of 50 ng pRL-CMV (Promega, Madison, WI, USA) expressing Renilla luciferase. Luciferase activity was determined after stimulation with LPS (1 μg/mL) for 12 h.

Chromatin immunoprecipitation assay. A ChIP assay was carried out according to the manufacturer's protocol (Santa Cruz Biotechnology) and a previous published report.[27] After cells were incubated with or without LPS (1 μg/mL) for 3 h, nuclear extracts were prepared and processed to precipitate specific DNA fragments with anti-Nrf2 antibody (H-300) or anti-Bach1 antibody (C-20). Isolated DNAs were semiquantified by real-time PCR using Power SYBR Green (Applied Biosystems) using the primers for human HO-1 MARE sites EN1 and EN2 described previously.[27]

Statistical analysis. Data are reported as the mean ± SEM. The paired *t*-test and Kruskal–Wallis test were used to test for differences. *P*-values <0.05 were considered to be statistically significant.

Results

Differential expression of HO-1, Nrf2, and Bach1 proteins in primary monocytes and leukemic cells. We first compared the expression levels of HO-1 among three human myelogenous leukemic cell lines (U937, HL60, and K562), circulating AML cells from 12 patients and ALL cells from four patients, and peripheral monocytes from four healthy donors (Table S1). Typical results are shown in Figure 1(A). Circulating $CD14^+$ monocytes in healthy donors expressed substantial amounts of HO-1, whereas cultured myelogenous leukemic cell lines expressed very little (Fig. 1A). The HO-1 levels in freshly isolated AML cells varied among patients (Fig. 1A). Among three morphological classes of AML, M4 cells tended to express HO-1 at high levels compared to other classes of AML cells and ALL cells, although the numbers of the test samples were not sufficient (Fig. S1).

To clarify the regulatory mechanism of the HO-1 expression, we next analyzed the transactivator Nrf2 and the repressor Bach1, both of which are involved in *HO-1* gene transcription. Both proteins, as well as HO-1, were found in the whole-cell lysates of monocytes and leukemic cells from most patients, but they were scarcely detected in the cultured cell lines (Fig. 1A,B). It was noted that the Bach1 levels in the primary cell samples were high even in the M4 class of AML cells, which expressed high levels of HO-1. To get insight into the functions of the two transcription factors, we separately analyzed the Nrf2 and Bach1 levels in cytoplasmic and nuclear fractions from AML cells and monocytes by immunoblotting. In both samples, Nrf2 was predominantly localized in the nuclei, whereas the majority of Bach1 protein was detected in the cytoplasmic fraction (Fig. 1C). The intracellular localization of Nrf2 and Bach1 proteins in U937 cells was difficult to determine because of the very low levels of expression (data not shown). The results suggested that the balance of nuclear levels, rather than the total cellular levels, of Bach1 and Nrf2 determines HO-1 expression, and that Nrf2 is essential for HO-1 expression.

Regulation of HO-1 expression by nuclear Nrf2 and Bach1 levels in response to LPS. LPS is known to affect HO-1 expression in leukemic cell lines and monocytes.[19,22–24] To understand the regulatory mechanism of HO-1 expression in leukemic cells, we compared the effect of LPS on HO-1 expression between the U937 cell line and $CD14^+$ primary monocytes. LPS

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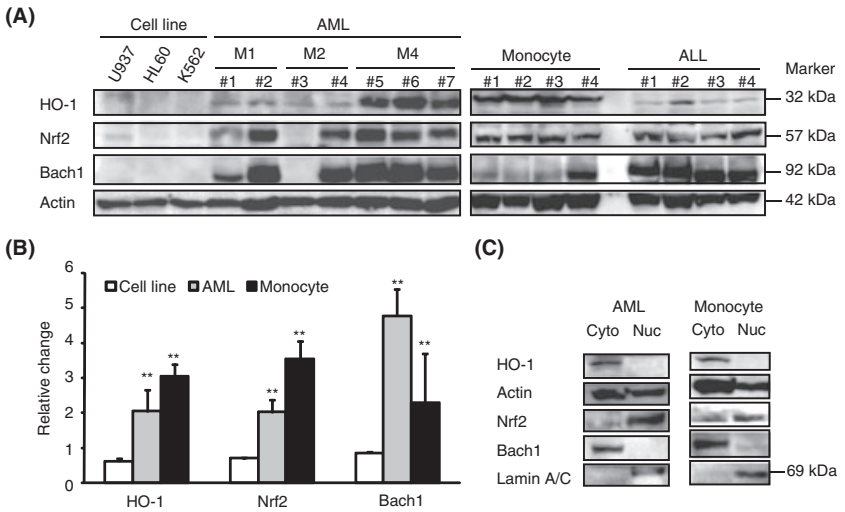

Fig. 1. Differential expression of heme oxygenase (HO)-1, nuclear factor erythroid-derived 2 related factor 2 (Nrf2), and BTB and CNC homology 1 (Bach1) proteins in primary monocytes and leukemic cells. (A) Expression levels of HO-1, Nrf2, and Bach1 were determined by immunoblotting techniques in: whole-cell lysates of leukemic cell lines (U937, HL60, and K562); circulating leukemic cells from patients with AML (M1, $n = 2$; M2, $n = 2$; and M4, $n = 3$) or ALL ($n = 4$); and peripheral monocytes from healthy donors ($n = 4$). (B) Data are given as the mean ± SEM; the U937 protein level was assigned a value of 1. Figure key describes: AML, cells obtained from 12 AML patients (M1, $n = 3$; M2, $n = 5$; and M4, $n = 4$); Cell line, myelogenous leukemic cell lines U937, HL60, and K562; Monocyte, monocytes from four healthy donors. $^{**}P < 0.01$ by paired t-test. (C) Fractionated cytoplasmic (Cyto) and nuclear (Nuc) extracts from AML cells (M1, patient #8) and $CD14^+$ monocytes were analyzed separately. Actin and Lamin A/C served as endogenous loading controls for cytoplasmic and nuclear proteins, respectively.

upregulated HO-1 in U937 cells, but this upregulation was not associated with an increase or decrease in Nrf2 or Bach1 levels in the whole cell lysates (Fig. 2A, left panel). In contrast, treatment of monocytes with LPS strongly suppressed HO-1 expression without altering the high expression levels of the two regulatory proteins (Fig. 2A, right panel). Analysis of nuclear levels of Nrf2 and Bach1 revealed that the LPS-induced HO-1 expression in U937 cells was associated with a clear increase in the Nrf2 level and a slight decrease in the Bach1 level in the nuclei, whereas the LPS-mediated reduction of HO-1 in monocytes was associated with a marked increase in the nuclear Bach1 level without any change in the Nrf2 level (Fig. 2B). Thus, LPS positively or negatively changed the HO-1 expression by increasing the nuclear level of either Nrf2 or Bach1, depending on the cell type, but without changing their total cellular levels.

Transcriptional activity of Bach1 and Nrf2 in the nuclei to the MARE sites EN1 and EN2 was examined by means of a ChIP assay. The assay revealed that Bach1 was highly bound to EN1 and EN2 sites in U937 cells under a resting condition (Fig. 2C, right panel), in accordance with previous reports.[15,26–28] However, the LPS treatment markedly reduced the binding level of Bach1 to the EN1 and EN2 sites (Fig. 2C, right panel), while it reciprocally increased that of Nrf2 (Fig. 2C, left panel). In contrast, Nrf2 rather than Bach1 bound predominantly to EN1 and EN2 sites in freshly isolated PBMC (Fig. 2D). Treatment of

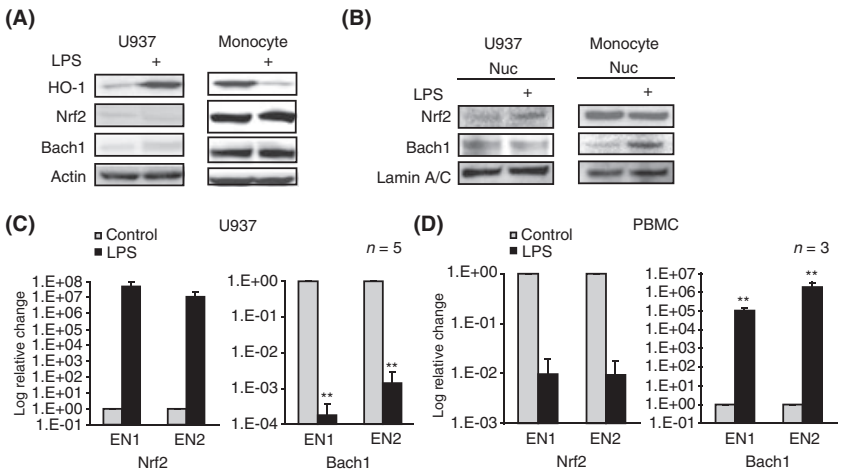

Fig. 2. Regulation of heme oxygenase (HO)-1 expression by nuclear factor erythroid-derived 2 related factor 2 (Nrf2) and BTB and CNC homology 1 (Bach1) levels in the nuclei in response to LPS. (A) Expression levels of HO-1, Nrf2, and Bach1 in U937 cells (left) and $CD14^+$ monocytes (right) were determined after 16 h in the presence or absence of LPS (1 μg/mL) stimulation. (B) After U937 cells and monocytes were stimulated with LPS for 3 h, nuclear fractions (Nuc) were prepared and subjected to immunoblotting for Nrf2 and Bach1. U937 cells (C) and peripheral blood mononuclear cells (PBMC) (D) were cultured with or without LPS for 3 h. Nrf2 and Bach1 binding to EN1 and EN2 was evaluated by ChIP assay. Values are given as the mean ± SEM for five sets of independent experiments with U937 and three sets with PBMC. $^{**}P < 0.01$ by paired t-test.

these cells with LPS completely reversed their binding levels to EN1 and EN2 (Fig. 2D). These results indicate that LPS treatment differentially regulates the binding levels of Nrf2 and Bach1 to the EN1 and EN2 sites, which determine HO-1 expression.

Change in cellular response to LPS for HO-1 expression by PMA treatment in leukemic cell lines. It has been suggested that HO-1 expression in myelomonocytic lineage cells is affected by a differentiation state.[5,38,39] PMA, a protein kinase C activator, induces differentiation of myelomonocytic leukemic cell lines into mature monocytes.[40] We found that treatment with PMA upregulated HO-1 expression in such cell lines, most typically in U937 (Fig. 3A). The upregulation was accompanied by an increase in the Bach1 level. Comparison between untreated and PMA-treated U937 cells showed that LPS strongly upregulated HO-1 at concentrations over 100 ng/mL in the former cells, but it suppressed HO-1 in the PMA-treated cells at 10–1000 ng/mL (Fig. 3A (lower panel), B). The downregulation of HO-1 by LPS became evident at 4 h and was almost completed within 12 h (Fig. 3C). Essentially the same results were obtained with K562 and HL60 cell lines (Fig. S2). Thus, the response of PMA-treated U937 cells to LPS was similar to that of primary monocytes (Fig. 2A). This implies that LPS produces opposite effects on the same cells depending on the cellular conditions controlled by external stimuli.

To find physiological factors with PMA-like activity, three cytokines were examined. γ-Interferon (IFN-γ) increased the Bach1 protein level in U937 cells (Fig. 3D). Like PMA-treated U937 cells, the HO-1 expression in U937 cells was markedly reduced by LPS in the presence of IFN-γ (Fig. 3E). Neither TNF-α nor IL-18 altered Bach1 expression levels (Fig. 3D), but both of them slightly increased HO-1 expression in response to LPS (Fig. 3E). These results suggest that LPS reduces HO-1 expression under Bach1-enriched conditions, but it enhances HO-1 expression under Bach1-reduced conditions. The data also imply that a signal induced by PMA or IFN-γ switches the cellular response to LPS for HO-1 expression.

Bach1-dependent transcriptional suppression of HO-1 expression at MARE in PMA/LPS-treated U937 cells. Transcriptional regulation of HO-1 expression was semiquantitatively determined by means of real-time PCR. In agreement with HO-1 protein levels, HO-1 mRNA gradually increased upon treatment with LPS in U937 cells, whereas LPS significantly suppressed mRNA levels in PMA-treated U937 cells for up to 9 h (Fig. 4A). The transcription inhibitor Act D completely abrogated the suppressive effect of LPS on HO-1 mRNA expression, indicating that LPS suppressed HO-1 expression at the transcriptional level (Fig. 4B). We further studied the transcription activity for HO-1 expression in PMA-treated U937 cells that had been transfected with a luciferase-expressing plasmid encoding the MARE EN1 region (pHO-1(-4.5k)) or with a plasmid lacking the MARE site (pHO-1(-4.0k)).[21] LPS attenuated luciferase activity in cells transfected with pHO-1 (-4.5k) but not in cells transfected with pHO-1(-4.0k), suggesting that the effect of LPS depended on the MARE (Fig. 4C). In PMA-treated U937 cells, nuclear translocation of Bach1 was also found 3 h after LPS stimulation (Fig. 4D), suggesting that Bach1 contributed to regulation of HO-1 expression.

We further examined the role of Bach1 in the regulation of HO-1 expression, using a Bach1 expression vector (pBach1)[33] and an siRNA for Bach1. Overexpression of Bach1 in U937 cells resulted in the reduction of HO-1 (Fig. 5A). In contrast, downregulation of Bach1 protein by siRNA caused a marked enhancement of HO-1 protein levels in a dose-dependent fashion (Fig. 5B). In this experiment, the Bach1 protein in U937 was detected by increasing the sensitivity of the immunoblotting assay. Moreover, transfection with Bach1 siRNA abrogated the LPS-mediated HO-1 reduction in PMA-treated U937 cells without affecting Nrf2 expression levels (Fig. 5C). Thus, the gene manipulation of Bach1 supports the hypothesis that Bach1 plays a key role in LPS-mediated HO-1 reduction.

Effects of knockdown of HO-1 and Bach1 expression on survival of AML cells. As shown in Figure 1(A), most cases of primary AML cells constitutively expressed Bach1 and HO-1 proteins. LPS treatment of primary AML cells reduced HO-1 expression, as seen in mature monocytes and PMA-treated U937 cells (Fig. 6A). The reduction of HO-1 was associated with translocation of Bach1 into the nuclei, suggesting a relative predominance of nuclear Bach1 to Nrf2 (Fig. 6B). To assess the roles of Bach1 and HO-1 on cell survival, we examined the effects of siRNAs for Bach1 and HO-1 on the survival of

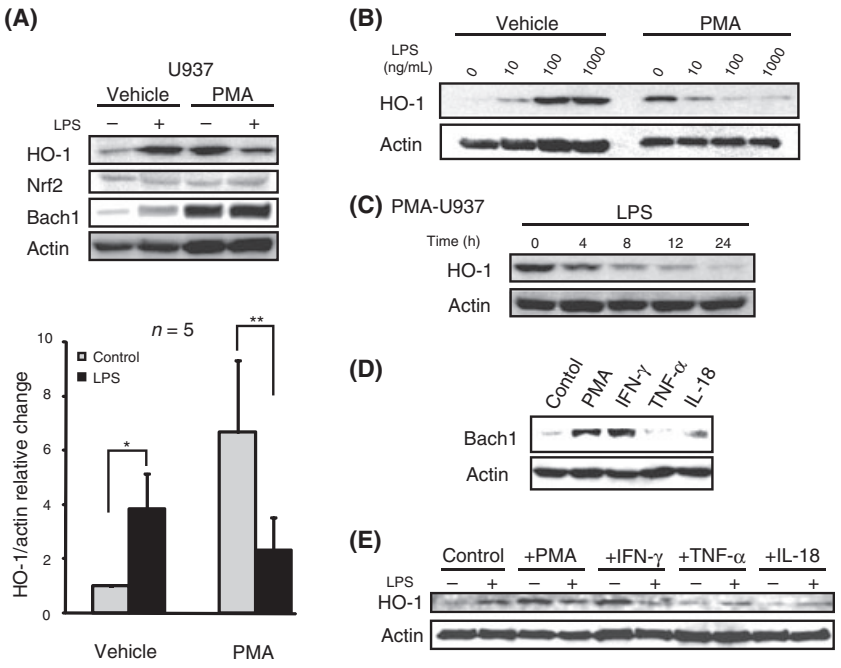

Fig. 3. Change of cellular response to LPS for heme oxygenase (HO)-1 expression by PMA treatment in leukemic cell lines. (A) U937 cells were pretreated with PMA (100 nM) or vehicle for 48 h. Expression levels of HO-1, nuclear factor erythroid-derived 2 related factor 2 (Nrf2), and BTB and CNC homology 1 (Bach1) were determined after a further 16 h of stimulation with LPS (1 μg/mL). In the lower panel, densitometric analysis of the blots is shown for five independent experiments (mean ± SEM). $^{**}P < 0.01$; $^{*}P < 0.05$ by paired *t*-test. (B) HO-1 expression was determined in PMA-treated and untreated U937 cells after treatment with the indicated concentrations of LPS. (C) HO-1 protein levels were determined at the indicated periods of time after LPS stimulation of PMA-treated U937 cells. (D) Expression levels of Bach1 were determined in U937 cells stimulated with PMA (100 nM), γ-interferon (IFN-γ; 100 ng/mL), tumor necrosis factor-α (TNF-α; 10 ng/mL), or interleukin-18 (IL-18; 100 ng/mL) for 48 h. (E) HO-1 expression was determined after the cells treated in (D) were further incubated with (+) or without (–) LPS.

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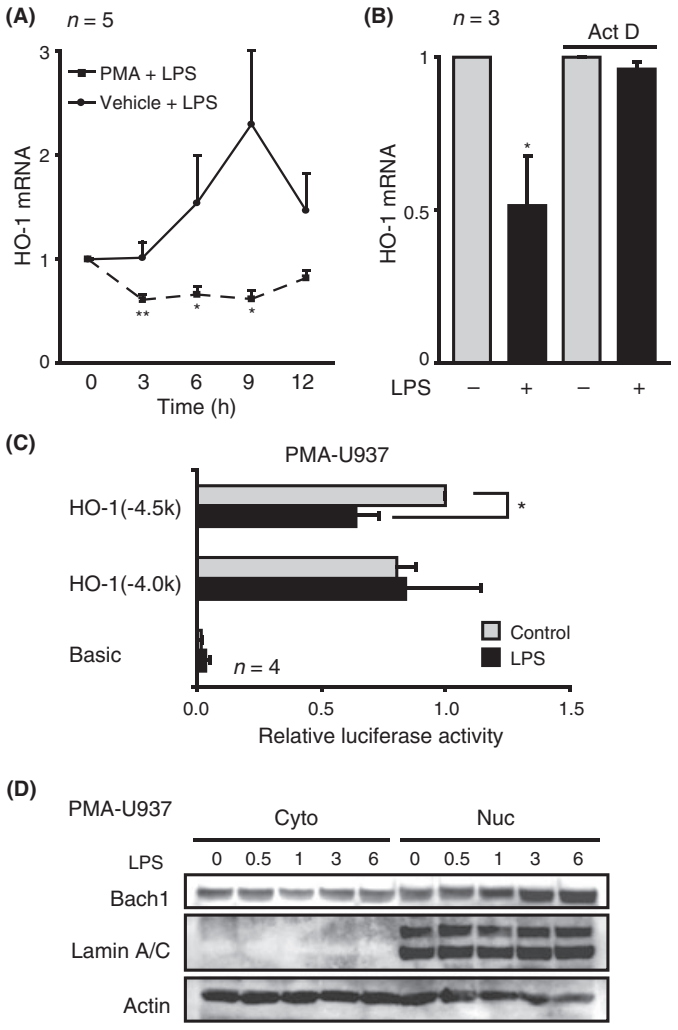

Fig. 4. BTB and CNC homology 1 (Bach1)-dependent transcriptional suppression of heme oxygenase (HO)-1 expression at Maf-recognition elements in PMA/LPS-treated U937 cells. (A) HO-1 mRNA levels were monitored by real-time PCR at the indicated time points after LPS (1 μg/mL) stimulation of PMA-treated (dotted line) or untreated (solid line) U937 cells. The expression level was standardized relative to the amounts of 18S rRNA. Values indicate relative quantity; the starting points of individual experimental series were assigned a value of 1. Data are given as the mean ± SEM for five independent experiments. $^{**}P < 0.01$; $^{*}P < 0.05$ by Kruskal–Wallis test. (B) HO-1 mRNA expression was determined in PMA-treated U937 cells before and after stimulation with LPS for 2.5 h in the presence or absence of actinomycin D (Act D; 5 μg/mL). Data are given as the mean ± SEM for three independent experiments; the basal expression level was assigned a value of 1. $^{*}P < 0.05$ by the paired *t*-test. (C) PMA-treated U937 cells were transfected with 2 μg plasmid pGL3 HO-1(-4.5k), pGL3 HO-1(-4.0k), or pGL3 basic vector, mixed with 50 ng PRL-CMV vector. Luciferase activity was determined after stimulation with LPS for a further 12 h. Values are given as the mean ± SEM of relative luciferase activity for four independent experiments. The unstimulated pGL3 HO-1(-4.5k) samples were assigned a value of 1. (D) PMA-treated U937 cells were stimulated with LPS for the indicated periods of time (0–6 h). Bach1 levels were examined in cytoplasmic and nuclear fractions by immunoblotting. Actin and Lamin A/C served as endogenous loading controls for cytoplasmic (Cyto) and nuclear (Nuc) proteins, respectively.

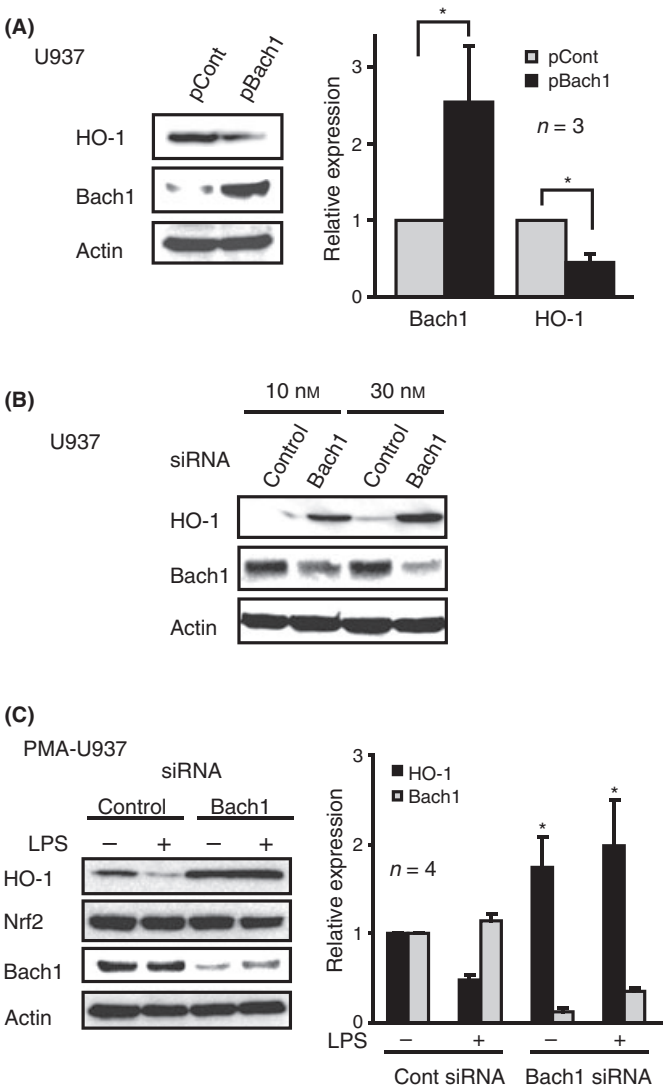

Fig. 5. Regulation of heme oxygenase (HO)-1 expression by manipulation of the BTB and CNC homology 1 (*Bach1*) gene. (A) Expression levels of Bach1 and HO-1 were examined in U937 cells transfected with 2 μg Bach1-encoding pcDNA3.1^{+} plasmid (pBach1) or control plasmid (pCont) for 6 h. Densitometrical analysis of three independent experiments is shown in the right panel (mean ± SEM). (B) After U937 cells were incubated with 10 or 30 nM Bach1 siRNA or negative control RNA for 16 h, Bach1 and HO-1 levels were examined by immunoblotting. The blot was analyzed after longer exposure to detect small amounts of Bach1. (C) After U937 cells were treated with 30 nM Bach1 or control RNA for 16 h, then with PMA for a further 24 h, they were further treated with or without LPS (1 μg/mL) for 16 h, followed by analysis of the HO-1 and Bach1 levels. Densitometric analysis of the blots is shown in the right panel. Values are given as the mean ± SEM of HO-1 or Bach1 levels relative to actin levels from four independent experiments.

primary AML cells. Each siRNA specifically suppressed the expression of corresponding proteins in AML cells (Fig. 6C). In addition, HO-1 was upregulated in AML cells treated with Bach1 siRNA (Fig. 6C). Under these conditions, transfection with Bach1 siRNA resulted in increased survival of primary AML cells, whereas knockdown of HO-1 led to reduced cell survival in the presence or absence of anticancer drug Ara-C (Fig. 6D). The relationship between Bach1 expression and the sensitivity to Ara-C was analyzed. Among the five cases of primary AML cells shown in Figure 1(A), there was no significant correlation ($r = -0.203$, $P = 0.743$) ($n = 5$) (data not shown). These data suggest that HO-1 protects primary AML cells from cell death, whereas Bach1 promotes cell death of AML by downregulating HO-1.

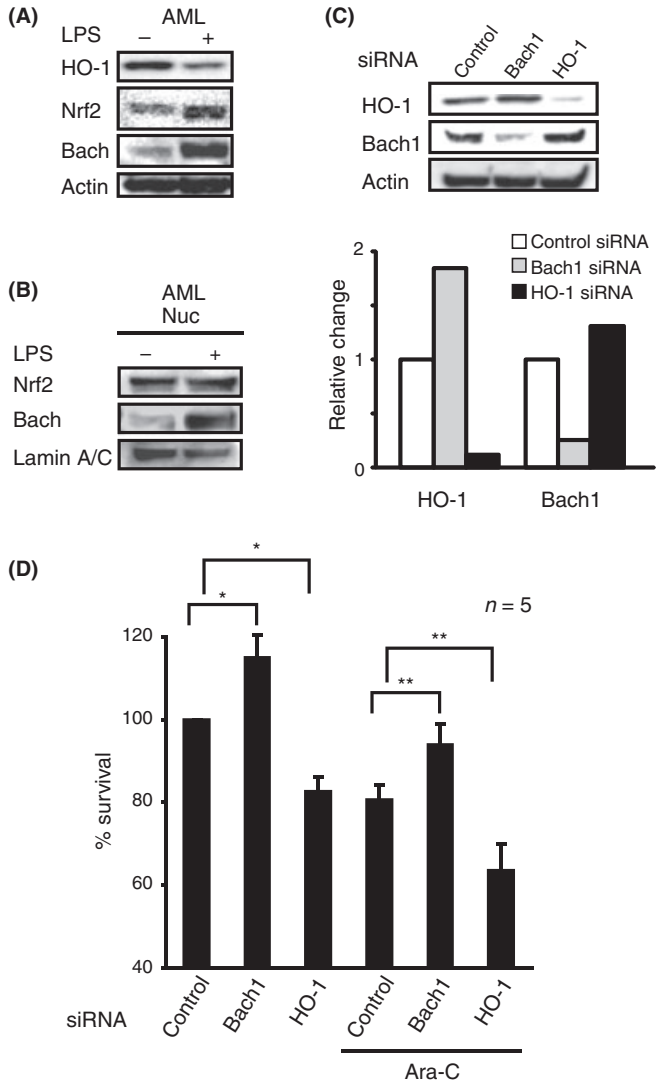

Fig. 6. Effects of knockdown of heme oxygenase (HO)-1 and BTB and CNC homology 1 (Bach1) expression on survival of AML cells. (A) Expression levels of Bach1, nuclear factor erythroid-derived 2 related factor 2 (Nrf2), and HO-1 in primary AML cells (M4, patient #6) were determined after 16 h in the presence or absence of LPS (1 μg/mL) stimulation. (B) After freshly isolated AML cells (M1, patient #8) were stimulated with LPS for 3 h, cells were fractionated into the nucleus (Nuc). Localization of Nrf2 and Bach1 was determined by immunoblotting. (C,D) AML cells were incubated with 50 nM Bach1 siRNA, HO-1 siRNA, or control RNA for 16 h, then with or without 10 μM cytosine arabinoside (Ara-C) for 24 h in the presence or absence of 10 μM Ara-C. (C) Representative immunoblots and densitometric quantitative data are shown for M1 cells (patient #2). (D) The cell viability for the individual culture conditions was evaluated by the tetrazolium method. Values are given as the mean ± SEM for five independent AML samples (M1, $n = 2$; M2, $n = 2$; and M4, $n = 1$; patients #2, 6, 8, 9, and 12), and the absorbance for control cells in the absence of Ara-C was assigned 100%. $^*P < 0.05$; $^{**}P < 0.05$ by paired *t*-test.

Discussion

The present study indicated that human primary AML cells, as well as normal monocytes from healthy donors, expressed HO-1 at appreciable levels. Among the morphological classes of AML cells, M4 cells tended to have higher HO-1 expression levels than did M1 or M2 cells. Cellular maturation seems to be one of the factors that determines HO-1 expression levels in the myelomonocytic cell lineage. HO-1 expression was also observed in ALL cells, irrespective of the presence or absence of the Philadelphia chromosome. It is noted that most cases of these primary leukemic cells expressed high levels of the transcriptional repressor Bach1. However, subcellular fractionation and subsequent immunoblotting of these primary cells indicated that Bach1 existed preferentially in the cytoplasm, whereas the transcriptional activator Nrf2 was localized mainly in the nuclei. In concordance with these results, the ChIP assay of PBMC showed the preferential binding of Nrf2 over Bach1 to the EN1 and EN2 regions. We also showed that the knockdown of *HO-1* gene expression suppressed cell survival and increased susceptibility to the anticancer drug Ara-C in AML cells, whereas the knockdown of Bach1 expression produced the opposite effects. These data suggest that HO-1 is important for growth of leukemic cells.

In contrast to primary leukemic cells, cultured leukemic cell lines such as U937, HL60, and K562 expressed little HO-1. Although Nrf2 and Bach1 proteins were barely detected in whole-cell lysates of the cell lines, the ChIP assay showed that the binding of Bach1 to EN1 and EN2 regions predominated over Nrf2 binding. However, after exposure to PMA, U937 cells expressed HO-1, Nrf2, and Bach1, as did primary leukemic cells and normal monocytes. It is well known that immature myelomonocytic cells differentiate to show morphologic, ultrastructural, and functional characteristics typical of the monocyte/macrophage lineage cells after treatment with PMA *in vitro*.[40] We confirmed the differentiation status of the PMA-treated U937 cells by determining monocyte/macrophage-specific CD14 mRNA expression using RT-PCR (data not shown). It is thought that PMA-treated U937 cells have at least partially acquired phenotypes of primary monocytes. Like PMA, IFN-γ stimulated the expression of both HO-1 and Bach1 in U937 cells. Previous studies with murine NIH3T3 fibroblasts have shown that oxidative stress promotes the translocation of Nrf2 into the nuclei and the export of Bach1 to the cytoplasm, resulting in *HO-1* gene transcription.[14,15,25] Based on our findings, it is supposed that leukemic cell lines have lost a signal transduction pathway, which is induced by PMA or IFN-γ, to express the three proteins during the establishment of these cell lines. In this regard, it is noted that STAT-3- and phosphoinositol-3 kinase-mediated signals are required for HO-1 induction by IL-10 in human and murine macrophages.[24]

It has been reported that LPS positively or negatively regulate HO-1, depending on cell types and species.[19,22–24,37,38] In the present study, LPS reduced the expression level of HO-1 by the relative predominance of nuclear Bach1 over Nrf2 in circulating normal monocytes and primary AML cells. Conversely, LPS upregulated HO-1 expression by the relative predominance of nuclear Nrf2 in U937 cells, but it reduced HO-1 expression in PMA-treated or IFN-γ-treated U937 cells. Treatment of U937 cells with a Bach1 cDNA or its siRNA indicated that the upregulation or downregulation of the *HO-1* gene is primarily determined by the nuclear Bach1 level. PMA-treated U937 cells were more similar to primary AML cells than to untreated U937 cells. It is likely that LPS upregulates HO-1 in the cells with low Bach1 expression, but it downregulates HO-1 in those with high Bach1 expression. These results also imply that the response to LPS is switched by a signal induced by PMA or IFN-γ. Moreover, IFN-γ, hypoxia, and desferrioxamine induce Bach1 mRNA expression, which is consistently associated with the repression of HO-1 expression,[41] although little is known about Bach1 expression mechanisms. We confirmed that LPS stimulation induced Bach1 mRNA expression rapidly in PMA-treated U937 cells, but suppressed expression in untreated U937 cells (data not shown). LPS is known to induce the Toll-like receptor signal transduction pathway, which might contribute to the transcriptional regulation of Bach1 mRNA. The detailed mechanisms by

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which Bach1 expression is regulated by LPS and PMA are important subjects of further investigation.

It has been suggested that Nrf2, as well as HO-1, is a potential therapeutic target for AML cells.[4,11] Our data show that Bach1 participates in an important way by controlling growth and survival of AML cells by regulating HO-1 expression. When the relationship between Bach1 expression and sensitivity to Ara-C was analyzed among different preparations of AML cells, there was no significant correlation (data not shown). However, the knockdown of Bach1 significantly increased the survival of AML cells, irrespective of the presence or absence of Ara-C, indicating that the Bach1 level is an important determinant for drug sensitivity in individual cases of AML cells. Thus, quantitative or functional upregulation of Bach1 is considered to be an alternative therapeutic strategy for leukemia. Bach1 exclusively regulates HO-1 and β-globin genes,[25] whereas Nrf2 is involved with various types of genes.[6] Therefore, we expect the approach using Bach1 to cause fewer adverse effects than that using Nrf2. For the Bach1-targeting therapy, it seems important to understand the mechanism by which LPS suppresses HO-1 expression in primary AML cells.

In summary, we showed that HO-1 expression levels in myelomonocytic lineage cells including AML cells is competitively regulated by the balance of the transactivator Nrf2 and the repressor Bach1 in the nuclei. The increase of nuclear Bach1 levels is expected to enhance the cytotoxic effects of some anticancer agents for AML cells. This strategy may also lead to a breakthrough in chemoresistance to conventional anticancer agents. Bach1 targeting is also promising for the suppression of inflammatory responses.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Expression levels of heme oxygenase (HO)-1, nuclear factor erythroid-derived 2 related factor 2 (Nrf2), and BTB and CNC homology 1 (Bach1) were determined by immunoblotting techniques in whole-cell lysates of three subtypes of AML cells (M1, $n = 3$; M2, $n = 5$; and M4, $n = 4$). The densitometric analysis of the blots is shown. Data are given as the mean ± SEM; the U937 protein level was assigned a value of 1.

Fig. S2. K562 and HL60 cells were pretreated with PMA (100 nM) or vehicle for 48 h. Expression levels of heme oxygenase (HO)-1, nuclear factor erythroid-derived 2 related factor 2 (Nrf2), and BTB and CNC homology 1 (Bach1) were determined after stimulation for a further 16 h with LPS (1 μg/mL).

Table S1. Characteristics of AML and ALL patients who participated in this study.

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