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Alkylating agent methyl methanesulfonate (MMS) induces a wave of global protein hyperacetylation: Implications in cancer cell death

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Abstract

Protein acetylation modification has been implicated in many cellular processes but the direct evidence for the involvement of protein acetylation in signal transduction is very limited. In the present study, we found that an alkylating agent methyl methanesulfonate (MMS) induces a robust and reversible hyperacetylation of both cytoplasmic and nuclear proteins during the early phase of the cellular response to MMS. Notably, the acetylation level upon MMS treatment was strongly correlated with the susceptibility of cancer cells, and the enhancement of MMS-induced acetylation by histone deacetylase (HDAC) inhibitors was shown to increase the cellular susceptibility. These results suggest protein acetylation is important for the cell death signal transduction pathway and indicate that the use of HDAC inhibitors for the treatment of cancer is relevant.

Keywords: Protein acetylation; Methyl methanesulfonate; Cell death; HDAC inhibitors

Protein acetylation is a dynamic posttranslational modification of lysine residues. Previous studies have shown that protein acetylation is involved in the regulation of diverse cellular processes [1]. The role of histone acetylation in the regulation of transcription is the most extensively studied example of protein acetylation. A number of proteins possessing histone acetyltransferase (HAT) activity have been identified, including CBP/p300, P/CAF, GCN5, TAF250, SRC1, ACTR, MOZ, and Tip60 [2]. The discovery of HAT enzymes has facilitated the identification of novel substrates for acetylation. Many of these substrates are transcriptional regulatory proteins, such as p53, E2F1, TFIIE, TCF, GATA1, HMGI(Y), ACTR, FOXO, and HIF1- α [2,3]. However, interestingly, recent studies have shown that HATs have substrates other than transcriptional regulatory proteins, such as WRN, the nucleus import factor importin-α, the cytoplasmic protein tubulin, Ku70, and PTEN [2,4–6], indicating that protein acetylation is involved in a wide range of cellular processes besides the regulation of transcription.

Acetylation modification is able to regulate protein function in a variety of ways. For example, the acetylation of the DNA-binding domain of transcription factors resulted in a decrease (HMGI(Y)) or increase (p53, GATA-1, MyoD) in DNA-binding activity [2]. Acetylation is also able to increase protein stability (p53, E2F) [2,7]. The acetylation of the HNF-4 and importin-α transcription factors resulted in increased nuclear accumulation of the proteins by inhibiting the nuclear export of the proteins [2]. Protein acetylation is also able to regulate protein–protein interactions (TCF, Ku70) [2,5]. If the target protein has an enzymatic activity, acetylation is able to modulate its enzymatic activity (PTEN) [6]. These results suggest that

Abbreviations: MMS, methyl methanesulfonate; HDAC, histone deacetylase; IR, γ Irradiation; NAC, *N*-acetylcysteine; GSH, glutathione.

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protein acetylation is involved in the regulation of various cellular processes, including the regulation of transcription, DNA replication, and apoptosis, by controlling diverse aspects of protein function, including DNA-binding ability, protein stability, protein–protein interaction, subcellular localization, and enzyme activity [1]. From these results, it has been proposed that protein acetylation may act as a mechanism for the transduction of cellular regulatory signals such as protein phosphorylation [8]. However, previous studies investigating the role of protein acetylation have been primarily focused on the effect of acetylation on the individual protein, thus the direct evidence for the involvement of protein acetylation in signal transduction is very limited. Given the importance of acetylation modification in many cellular processes, the understanding of the exact role of protein acetylation in cellular signal transduction in response to genotoxic stress is an important issue.

Through molecular and biochemical studies in previous decades, it is well established that genotoxic stresses, including γ and UV irradiation, hydroxyurea, H_2O_2, and alkylating agents, induce intracellular signaling pathways via posttranslational modifications such as protein phosphorylation. Methyl methanesulfonate (MMS) is a highly toxic DNA-alkylating agent. MMS methylates the DNA bases and causes DNA damage leading to strand breaks, chromosome breaks, micronucleus formation, and cell death [9]. MMS has been used for many years as a representative DNA damaging agent in the field of cancer research, and it has also been used in the treatment of several human cancers [10]. However, the exact mechanism for MMS cytotoxicity is not fully understood. In this study, we found that MMS induces a robust and reversible acetylation of global cellular proteins. Functional analysis using various cancer cells revealed that the level of MMS-induced acetylation was strongly correlated with cellular susceptibility to MMS.

Materials and methods

Cell culture and treatments. HEK293, HCT116, HepG2, U2OS, ATM-deficient (GM09607), and wild type fibroblast (GM00637) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Gibco). For the genotoxic treatment, cells were treated with γ irradiation (IR) by using a ^{131}Cs source, UV by using a germicidal UV lamp and UV radiometer (UVX-25, Ultra-Violet Products Ltd), H_2O_2 or MMS (Sigma, St. Louis, MO, USA) with the indicated dose.

To examine the effect of reducing agents, glutathione (GSH, 15 mM) or *N*-acetylcysteine (NAC, 15 mM) was pretreated for 2 h and subsequently cultured in the presence of MMS. To examine the effect of HDAC inhibitors, Trichostatin A (TSA, 300 ng/ml) and nicotinamide (NAM, 10 mM) were pretreated for 4 h and subsequently treated with or without MMS for 2 h. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), GSH, NAC, TSA, and NAM were purchased from Sigma–Aldrich Co., Ltd.

Western blot analysis. To measure protein acetylation, cells harvested after treatment were lysed in RIPA buffer and subjected to Western blot analysis as described previously [11] by using rabbit polyclonal anti-acetylated-lysine antibody (Cell Signaling Technology Inc., Danvers, MA, USA). Actin protein was also monitored using anti-actin antibodies (Sigma) serving as an internal loading control.

Isolation of cytoplasmic and nuclear protein fractions . To prepare nuclear and cytoplasmic fractions, HEK293 cells were treated with 200 μg/ml of MMS for 2 h and then harvested. The cytoplasmic and nuclear fractions were prepared as described previously [12]. Both cytoplasmic and nuclear fractions were analyzed by Western blotting using anti-acetylated lysine and anti-actin antibodies.

MMS sensitivity assay. Four human cancer cell lines (HEK293, U2OS, HCT116, and HepG2) were treated with 100 or 200 μg/ml MMS for 1 h. Surviving cells were determined by Trypan blue exclusion 48 h after treatment. Survival experiments were performed in duplicate or triplicate and the data were presented as mean values with standard deviation.

Isolation of human peripheral blood monocytes. Peripheral blood leukocyte concentrates were donated by healthy young donors and leukemia patients. Peripheral blood mononuclear cells were separated as described previously [13]. Isolated monocytes were treated without or with MMS for 2 h and cell lysates were prepared for the Western blotting as described above by using anti-acetylated lysine and anti-Erk antibodies (Cell Signaling Technology Inc.).

Results

MMS induces a robust and reversible hyperacetylation of cellular proteins in the early phase

In order to explore the role of protein acetylation modification in the process of signal transduction, we examined the alteration in the level of protein acetylation after various genotoxic treatments, including γ-ray (IR) and UV irradiation, H_2O_2, and methyl methanesulfonate (MMS) in HEK293 cells. The level of acetylation of total lysates was measured with Western blotting using anti-acetylated-lysine (AcK) antibodies. As shown in Fig. 1A, we observed a very strong acetylation signal corresponding to proteins of a wide range of molecular weights in the MMS-treated sample, while a weak acetylation signal was observed in the H_2O_2-treated lysates (Fig. 1A). In contrast, protein acetylation was not observed in both IR- and UV-treated cell lysates, even after long periods of exposure (data not shown). To test whether other alkylating agents are also able to induce protein acetylation, we treated HEK293 cells with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS), and found both MNNG- and EMS-induced protein acetylation although the strength of acetylation was weaker than MMS (Fig. 1B). Since MMS showed the greatest capacity to induce protein acetylation, we decided to investigate this potentially interesting phenotype in order to explore the role of protein acetylation in signal transduction.

To explore MMS-induced acetylation, we first examined the time- and dose-dependent kinetics of protein acetylation. The analysis of the time-dependent kinetics of protein acetylation showed that protein acetylation began to appear at 1 h after MMS treatment and peaked at 2 h after treatment (Fig. 1C). The acetylation level gradually decreased thereafter and returned to the normal level 12 h later. The results of the dose-dependent kinetics analysis showed that MMS was able to induce protein acetylation at a concentration as low as 50 μg/ml (Fig. 1D). These

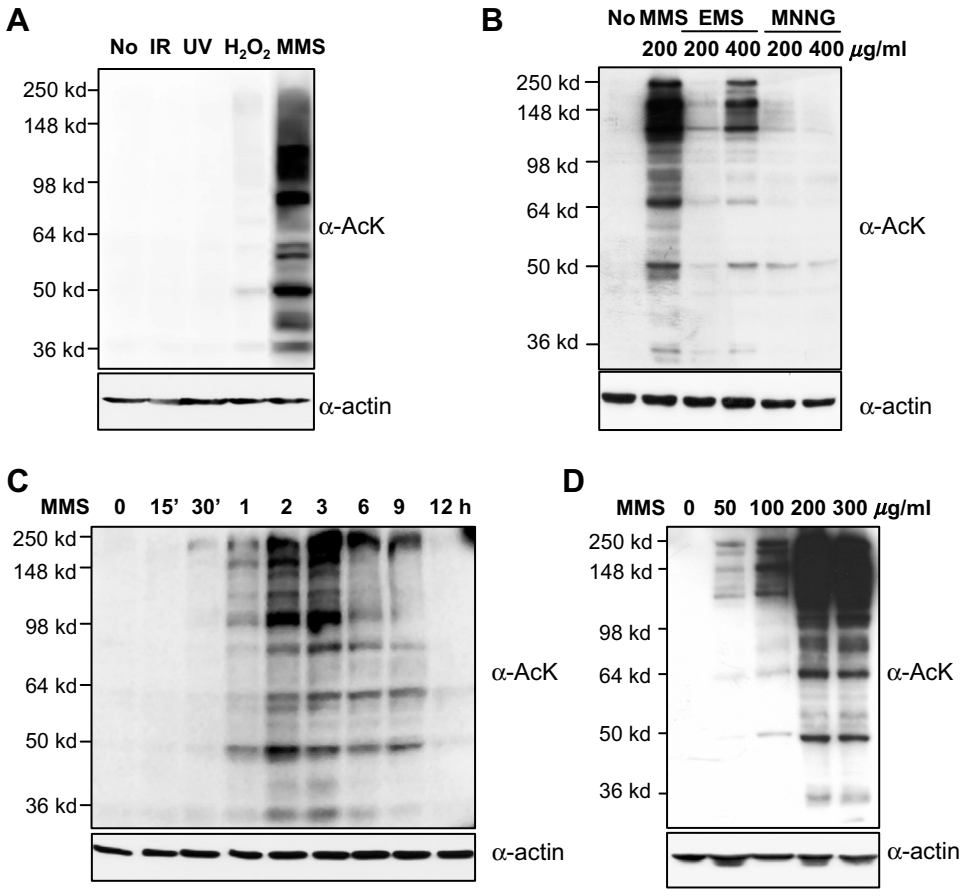

Fig. 1. MMS induces a wave of cellular protein hyperacetylation. (A) HEK293 cells were untreated or treated with γ irradiation (IR; 10 Gy), UV (20 J/m^2), H_2O_2 (1 mM) or MMS (300 μg/ml) and harvested 2 h later. Whole cell extracts were prepared and analyzed by Western blotting using anti-acetylated-lysine antibodies and anti-actin antibodies. (B) HEK293 cells were treated with the indicated concentrations of MMS, MNNG or EMS and harvested 2 h later. (C) HEK293 cells were untreated or treated with 300 μg/ml of MMS and harvested at the indicated time points. (D) HEK293 cells were untreated or treated with 50, 100, 200, or 300 μg/ml of MMS and harvested 2 h later. Acetylated proteins and actin were examined as described above.

results suggest that MMS treatment induces a reversible protein hyperacetylation during the early phase of the cellular response.

MMS induces hyperacetylation of global cellular proteins

The results of the Western blot analysis showed that the acetylated proteins were distributed over a wide range of molecular weights. This acetylation pattern raised the possibility that MMS induced the acetylation of both nuclear and cytoplasmic proteins. To examine this possibility, we analyzed protein acetylation with the cytoplasmic and nuclear fraction. Western blotting result indicated that the cytoplasmic proteins and nuclear proteins were fractionated successfully (Fig. 2A). Using these fractions, we found that protein acetylation was observed in not only nuclear but also cytoplasmic fractions. In addition, the difference in the pattern of the acetylation bands between the nuclear and cytoplasmic fractions suggests that a different subset of proteins from the cytoplasm and nucleus are acetylated upon MMS treatment.

NAC and GSH partially inhibit MMS-induced acetylation

Since it is known that MMS activates the DNA damage checkpoint by inducing DNA double strand breaks (DSB) [9], we examined whether the ATM pathway, which is a major signal pathway for the DNA damage checkpoint induced by DSB [14], is required for MMS-induced acetylation. However, MMS treatment induced protein acetylation in both $AT^{+/+}$ and $AT^{-/-}$ fibroblasts, indicating that the ATM pathway is not involved in MMS-induced acetylation (Fig. 2B).

It has been shown that MMS induced oxidative stress and decreased the intracellular levels of glutathione (GSH) [15]. To assess the role of oxidative stress on MMS-induced acetylation, we examined the effect of pretreatment of cells with a reducing agent, GSH or *N*-acetylcysteine (NAC). As shown in Fig. 2C, pretreatment with NAC and GSH significantly decreased the level of MMS-induced acetylation. These results suggest that the oxidative stress produced by MMS treatment may be important in MMS-induced acetylation, at least in part.

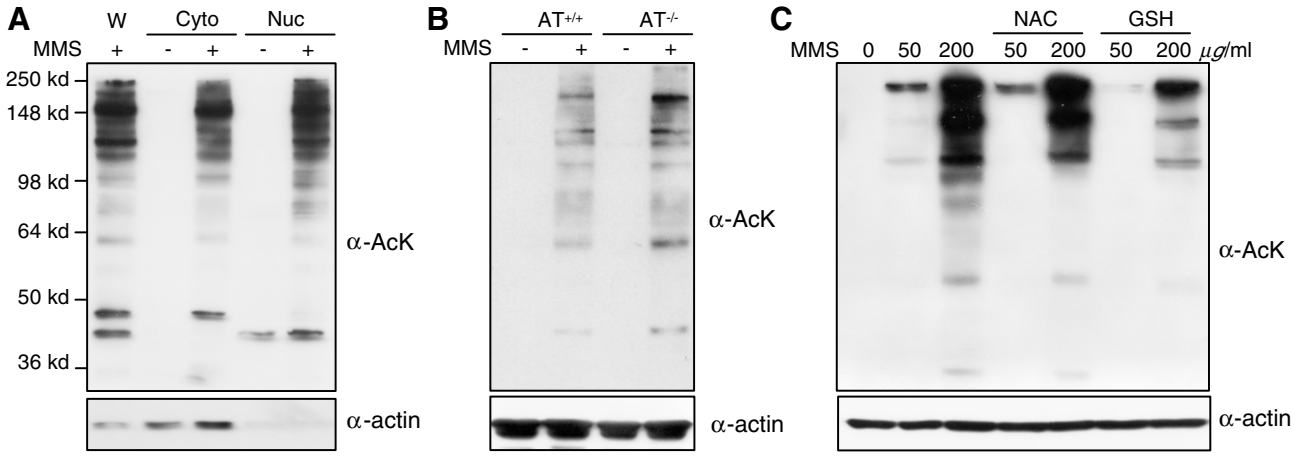

Fig. 2. MMS induces hyperacetylation of both cytoplasmic and nuclear proteins. (A) Nuclear (Nuc) and cytoplasmic (Cyto) fractions were prepared from HEK293 cells with or without MMS treatment as described in Materials and methods. Thirty micrograms of proteins was subjected to Western blotting using anti-acetyl-lysine antibodies and anti-actin antibodies. The whole cell extract (W) was used as a control. (B) AT-deficient ($AT^{-/-}$) and wild type fibroblasts ($AT^{+/+}$) were untreated or treated with 300 μg/ml of MMS for 2 h and harvested. The acetylated protein and actin levels were examined as described above. (C) HEK293 cells were pretreated with 15 mM of *N*-acetylcysteine (NAC) or glutathione (GSH) for 2 h and subsequently cultured in the presence of the indicated concentrations of MMS. The acetylated protein and actin levels were examined as described above.

MMS-induced acetylation is correlated with the susceptibility of cancer cells

To address the physiological relevance of MMS-induced hyperacetylation, we examined the level of protein acetylation using four different cancer cell lines. We found that HEK293 and HCT116 cells showed strong acetylation upon MMS treatment, while HepG2 and U2OS cells showed much weaker acetylation in response to MMS (Fig. 3A). MMS is a highly potent cytotoxic reagent and treatment with MMS induces extensive cell death [9]. To examine whether the hyperacetylation of the protein is important for the cellular sensitivity to MMS, we treated HEK293, HCT116, HepG2, and U2OS cells with various doses of MMS and measured the survival of these cells following treatment. As shown in Fig. 3B, U2OS and HepG2 cells showed considerable resistance up to the level of 200 μg/ml of MMS treatment. However, the HEK293 and HCT116 cells showed marked hypersensitivity to MMS.

To evaluate the potential link between protein acetylation and the cytotoxicity of MMS, we examined whether the modulation of the level of MMS-induced protein acetylation would affect the susceptibility of the cells to MMS treatment. In order to modulate the level of MMS-induced acetylation, we used trichostatin A (TSA) and nicotinamide (NAM), which are potent inhibitors for class I/II and class III HDACs, respectively [16]. As shown in Fig. 3C, the combined treatment of TSA and NAM significantly increased the level of MMS-induced acetylation, while TSA and NAM treatment alone did not have significant effects on the level of acetylation. If protein acetylation is critical for MMS-induced cell death, cellular susceptibility should increase by the combined treatment of TSA and NAM with MMS. Indeed, we observed that treatment of HDAC inhibitors significantly increased the susceptibility to MMS in HepG2 and U2OS (Fig. 3D).

Cancer cells showed increased acetylation in response to MMS

The abnormality of protein acetyltransferase and HDACs has been observed in various human cancers [16]. We examined the level of protein acetylation after MMS treatment using monocytes isolated from healthy young adults and those from patients with leukemia in order to assess whether the signal mechanism for MMS-induced hyperacetylation is altered during tumorigenesis. Since we found that the level of actin was decreased in human leukemia monocytes, Western blot analysis was carried out using anti-Erk antibody to confirm the equal loading of protein lysates (data not shown). As shown in Fig. 4, the level of protein acetylation upon MMS treatment was significantly higher in the human leukemia monocytes than in the healthy monocytes. These data indicate that the signal pathway for MMS-induced acetylation is largely dysregulated during tumorigenesis.

Discussion

Reversible posttranslational modification is a representative means for regulating protein function during signal transduction of extracellular stimuli. Protein phosphorylation is the most extensively studied type of modification that regulates protein activity in order to convey a cellular regulatory signal. For example, the early wave of tyrosine phosphorylation is often regarded as the induction of a signal transduction pathway in response to various growth and differentiation stimuli [17]. The evidence presented in the present study indicates that protein acetylation induced

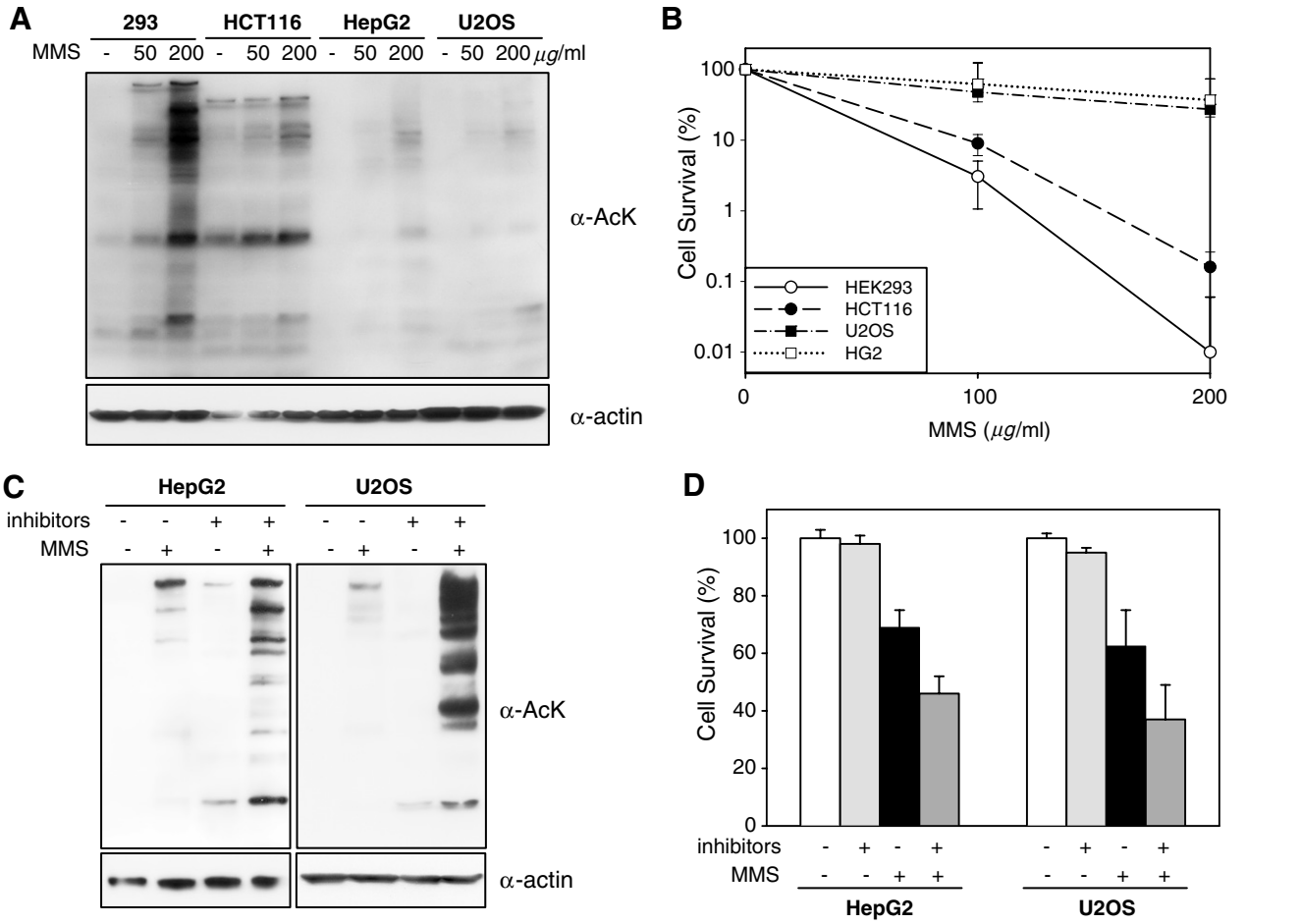

Fig. 3. MMS-induced acetylation is correlated with cellular susceptibility to MMS in cancer cells. (A) Four different cancer cell lines were treated with the indicated concentrations of MMS for 2 h and then harvested. The acetylated protein and actin levels were examined using anti-acetylated-lysine antibodies and anti-actin antibodies, respectively. (B) Cells were treated with 100 or 200 μg/ml of MMS for 1 h and surviving cells were counted by Trypan blue exclusion assay using hematocytometry. Error bars indicate standard deviation. (C) HepG2 and U2OS cells were untreated or pretreated with 300 ng/ml of TSA and 10 mM of NAM for 4 h (inhibitors) and subsequently treated with or without 200 μg/ml of MMS for 2 h. The acetylated protein and actin levels were examined as described above. (D) Survival of HepG2 and U2OS cells after treatment of 200 μg/ml of MMS for 1 h without or with pretreatment of HDAC inhibitors was examined as described in (B).

upon MMS treatment is likely to play a role in signal transduction. We showed that an alkylating agent, MMS, induced a robust wave of global cellular protein hyperacetylation. In the time-dependent kinetics analysis, MMS-induced hyperacetylation peaked within 2 h of treatment and totally disappeared 12 h later. This pattern suggests that the MMS-induced acetylation is reversible and that it only occurred during the early phase of the cellular response to MMS treatment, which is a typical feature of the involvement in cellular signal transduction. Importantly, the level of acetylation was strongly correlated with the cellular susceptibility to MMS. Furthermore, the modulation of MMS-induced acetylation using HDAC inhibitors, TSA and NAM, significantly increased the cellular susceptibility, indicating that MMS-induced acetylation is not merely a consequence of the cellular response, but that it is an important regulatory event in the cellular response to MMS. Thus, our results provide evidence for the notion that protein acetylation serves as a regulatory modification like protein phosphorylation [8].

Previous studies demonstrated that the activity of acetyltransferase was regulated by extracellular signals. For example, ACTR, the co-activator of the nuclear receptor, is acetylated in response to hormonal signal, and it mediates the hormone-induced repression of nuclear receptor function [18]. In addition, the HAT activity of CBP is increased at the G1-S boundary [19]. More interestingly, it has been shown that the acetyltransferase activity of GCN5 is inhibited through phosphorylation by DNA-dependent protein kinase (DNA-PK) [20]. These studies indicate that extracellular stimuli, such as proliferation signals and DNA damage, can modulate cellular acetyltransferase activity. Consistent with this notion, our results suggest that the intracellular acetyltransferase activity is elevated by MMS treatment during the early phase of the cellular response. The further increase of protein acetylation by MMS treatment in the presence of the HDAC inhibitors (Fig. 3C) indicates that protein acetylation upon MMS is not due to inhibition of HDAC activity but due to activation of acetyltransferase activity.

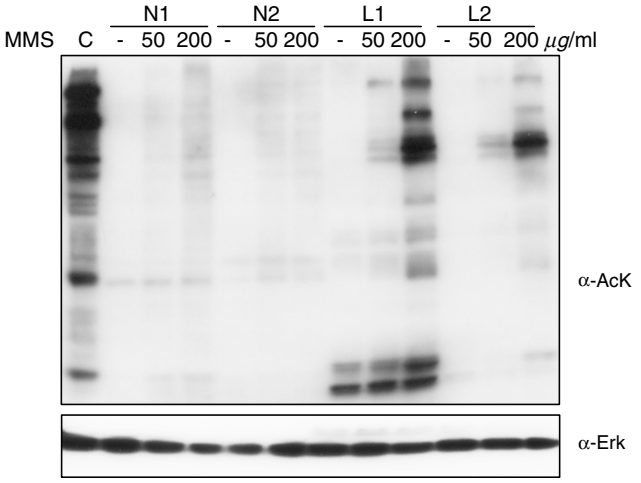

Fig. 4. Human leukemia monocytes showed enhanced acetylation in response to MMS treatment when compared with healthy monocytes. Human peripheral blood monocytes isolated from young healthy donors (N1 and N2) and patients with leukemia (L1 and L2) were treated with 50 or 200 μg/ml of MMS for 2 h and then harvested. The acetylated protein and actin levels were examined using anti-acetylated-lysine antibodies and anti-Erk antibodies, respectively.

We also noted that both nuclear and cytoplasmic proteins were intensively acetylated upon MMS treatment (Fig. 2A). To date, the majority of substrates for protein acetylation have been nuclear proteins. However, several proteins in the cytoplasm were found to be acetylated. The cytoplasmic proteins tubulin, Ku70, and PTEN were shown to be substrates of acetyltransferase [2,6]. The fact that MMS treatment induced the hyperacetylation of both cytoplasmic and nuclear proteins strongly suggests the existence of a cytoplasmic acetyltransferase enzyme. Cohen et al. showed that CBP proteins accumulated in the cytoplasm in response to UV irradiation [5]. Therefore, it is tempting to speculate that CBP is a major acetyltransferase for MMS-induced acetylation. However, neither the overexpression of CBP/p300 nor the co-treatment of the CBP inhibitor curcumin with MMS affected the level of protein acetylation, which suggests that CBP/p300 is not responsible for the MMS-induced protein acetylation (data not shown). Thus, the cytoplasmic acetyltransferase corresponding to MMS-induced acetylation remains to be identified, and this could provide important insights into the understanding of the signaling pathway of MMS-induced protein acetylation.

Although we focused our investigations on MMS-induced acetylation because of the advantages associated with the detection of protein acetylation, it is important to remember that H_2O_2 also induced protein acetylation. Oxidative stress is likely to be a strong inducer of protein acetylation since reducing agents, such as GSH and *N*-acetylcysteine (NAC), significantly inhibited MMS-induced acetylation. In addition, we could not exclude the possibility that the other genotoxic agents, such as IR and UV, also induced the acetylation of cellular proteins. In fact, it has been shown that a number of important regulatory proteins were acetylated upon IR and UV irradiation. The tumor suppressor protein p53 is acetylated after IR and UV irradiation [21]. The UV-induced acetylation of Ku70, pRb, and WRN was also reported [4,5,22]. Thus, the failure of acetylated protein in IR- and UV-treated lysates is possibly due to the limitation of detection sensitivity of current antibodies.

Aberrant acetylation and deacetylation activity has been observed in various diseases, including solid tumors and leukemias [16,23]. Recently, HDAC inhibitors have become considered as novel candidates for anti-cancer drug, and several HDAC inhibitors are being tested in clinical trials as monotherapy or in combination trials with other cytotoxic agents, such as carboplatin and paclitaxel [16]. We showed that cancer cells exhibiting a higher level of acetylation upon MMS treatment were more sensitive to MMS-induced cell death and that peripheral mononuclear cells derived from patients with leukemia showed higher levels of MMS-induced acetylation than monocytes from healthy subjects. These results indicate that HDAC inhibitors may sensitize cancer cells to genotoxic stress-induced cell death. In addition, given the correlation between enhanced acetylation and cellular susceptibility, our results suggest that the combination treatment of HDAC inhibitors with chemotherapeutic drugs would be useful for the treatment of human cancers.

Acknowledgments

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