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ORIGINAL ARTICLE

Oxidative stress enhances granulocytic differentiation in HL 60 cells, an acute promyelocytic leukemia cell line

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Abstract

This paper studied the effects of physiologically available oxidants on HL 60 differentiation induced by all-trans retinoic acid (ATRA) or dimethyl sulfoxide (DMSO). Hydrogen peroxide (15 μM) and taurine chloramine (200 μM) induced HL 60 differentiation, which was detected by CD11b expression and superoxide production. *Cd11b* and *p67^{phox}* mRNA expression was also augmented by these oxidants. In contrast, reducing chemicals, such as dithiothreitol, 2,3-dimercapto-1-propanol and N-acetylcysteine inhibited CD11b expression. Notably, DMSO inhibited methionine sulfoxide reductase activity, induced heme oxygenase-1 (*ho-1*) mRNA and enhanced oxidant-induced cell death, which indicated that DMSO intensified oxidative stress. After the addition of oxidants, *ho-1* expression preceded the *cd11b* expression. Vicinal dithiol-reactive phenylarsine oxide (50 nM) also increased CD11b expression induced by DMSO or ATRA. These observations suggested that oxidative stress enhanced granulocytic differentiation of HL 60 cells and that leukaemic cell differentiation was affected by cellular redox status.

Keywords: *Cell differentiation, leukemia, protein oxidation, thiol, methionine sulfoxide*

Introduction

Induction of cell differentiation is a unique therapeutic strategy for malignant neoplasm, and the most successful example is all-trans retinoic acid (ATRA)-induced differentiation of acute promyelocytic leukaemia (APL). Almost all APL cases have translocation of chromosome 15 and 17, which generates promyelocytic leukaemia-retinoic acid receptor α fusion gene [1]. The fusion gene product inhibits differentiation, which can be reversed by excess dose of ATRA [2]. Introduction of ATRA dramatically improved the outcome of APL, which once was the least curable leukaemic sub-type, and is now the best curable one [1]. However, ATRA is not effective to all types of leukaemia and the new approach is needed to expand cancer therapy by cell differentiation.

HL 60 is an APL-derived cell line and it differentiates into granulocyte, monocyte and macrophage when stimulated by ATRA, 1α, 25-dihydroxy vitamin D_3 and phorbol 12-myristate 13-acetate (PMA), respectively. Dimethyl sulphoxide (DMSO) also induces cell differentiation in several tumour cell lines, such as HL 60, K562 (from chronic myelogenous leukaemia blast crisis) [3] and murine Friend leukaemia cells [4], although the mechanism is still obscure. DMSO induces granulocytic differentiation in HL 60 cells, and the differentiated cells express CD11b and NADPH oxidase components, such as $p67^{phox}$, $gp91^{phox}$ and $p47^{phox}$ [5,6]. Functionally, the differentiated cells produce superoxide anion when stimulated by PMA.

Oxidative stress has been reported to affect cell differentiation. During the repair process after neuronal injury, oxidative stress directs neural progenitor cells to astroglial differentiation [7]. Oxidative stress also promotes human T-cell differentiation toward T helper 2 phenotype [8]. Reducing chemical, N-acetylcysteine

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(NAC), suppressed PC12 cell differentiation [9]. Under oxidative stress, protein thiol group in Cys and thio-ether group in Met are susceptible to oxidative modification, which has a regulatory role in cell signalling [10,11]. Cys oxidation of transcriptional regulatory protein Keap1 results in the activation of nuclear factor erythroid 2-like 2 (Nrf2), a transcription factor that regulates stress-response genes [12,13]. Actually, oxidative stress induces several genes under the control of Nrf2, and the most famous example is heme oxygenase-1 gene (*ho-1*). The HO-1 protein has antioxidant function, and *ho-1* gene expression is often used as a marker for oxidative stress [14].

Met oxidation to methionine sulphoxide (MetO) is another frequent protein modification under oxidative stress [15], and may alter cellular functions, such as NK-κB activation [16,17] or ion channel function [18]. MetO is reduced back to Met by methionine sulphoxide reductase (MSR), which consists of several isozymes that reduce protein-bound as well as free methionine sulphoxide using thioredoxin (Trx) as a reducing substrate [19].

We report here that oxidative stress by physiologically attainable oxidants, such as H_2O_2 and taurine-chloramine (Tau-Cl), stimulate HL60 differentiation by ATRA or DMSO. In addition, DMSO works as oxidative stress to the cells and oxidative stress is likely to be involved in DMSO-induced HL60 differentiation. These findings implicate that leukocyte differentiation is affected by cellular redox status and that leukaemia therapy may be improved by redox control.

Materials and methods

Chemicals

All-trans retinoic acid (ATRA), phenylarsine oxide (PAO), 2,3-dimercapto-1-propanol (BAL) dithiothreitol (DTT) and nitroblue tetrazolium (NBT) were obtained from Wako pure chemical (Osaka, Japan). Aminophenyl fluorescein (APF) was from Sekisui Medical (Tokyo, Japan). Alexa Fluor 488-labelled anti-human CD11b antibody was from BioLegend (San Diego, CA). RPMI 1640 medium (R8758), DMSO, L-methionine sulphoxide (MetO) and N-acetylcysteine (NAC) were from Sigma (St. Louis, MO). L-glutamin, sodium pyruvate and Dulbecco's phosphate buffered saline (D-PBS(-)) were from Life Technologies (Invitrogen, Carlsbad, CA). Taurine-chloramine (Tau-Cl) and monochloramine (NH_2Cl) were prepared as described previously [20]. Briefly, ice-cold 10 mM taurine or NH_4Cl in H_2O were quickly mixed with NaOCl and the generated chloramine derivatives were measured by absorption spectra. Typically, 4–5 mM Tau-Cl and NH_2Cl were obtained. About 10 mM H_2O_2 was prepared by the dilution of 30% H_2O_2 (from Wako) with H_2O and the concentration was measured by the absorption spectra [21]. All other chemicals are of analytical grade or better.

Cell culture and treatments

HL60 cells (JCRB0085) were obtained from Health Science Research Resources Bank (Osaka, Japan). The culture medium was RPMI 1640 supplemented with 10% FBS, 2 mM L-Gln and 1 mM sodium pyruvate. Cells were cultured in a humidified CO_2 incubator at 37°C with 5% CO_2 and used in their logarithmic growth phase.

For experiments, HL 60 cells were suspended in a fresh culture medium at 2×10^5 cells/ml. Cell differentiation was induced by DMSO (1.2%(v/v)) or ATRA (0.5 μM). Then the following chemicals were added at the indicated final concentrations: H_2O_2 (15 μM), Tau-Cl (200 μM), DTT (50 μM, added twice at 0 and 24 h), BAL (15 μM, added twice at 0 and 24 h), NAC (200 μM, added twice at 0 and 24 h) and PAO (20, 50 nM). The cells were cultured for 48 h and harvested for the following experiments.

Flow cytometry

Collected cells were washed once with D-PBS(−) + 0.2% (w/v) bovine serum albumin and reacted with Alexa Fluor 488-labelled anti-human CD11b antibody for 1 h on ice with occasional mixing. Then the cells were washed again and suspended in D-PBS(−) containing 0.5 μg/ml propidium iodide (PI) and analysed by a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter, Miami, FL). PI(+), PI(−) CD11b(+) and PI(−) CD11b(−) cells were considered as dead, differentiated and undifferentiated cells, respectively.

NBT reduction assay

Superoxide production capacity was studied by NBT reduction after PMA stimulation. Collected cells were washed once with fresh culture medium, then suspended in a buffer containing 0.33 μM PMA, 0.5 mg/ml NBT, 5 mM phosphate buffer (pH 7.4) and 70 mM NaCl and incubated at 37°C for 15 min. Superoxide reduces NBT to formazan, which stains the cells blue-black. Then the cells were counted under a light microscope.

Quantitative PCR

Total RNA was extracted from the cells using RNeasy mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. RNA concentration was measured by a spectrophotometer and cDNA was prepared from the equal amount of RNA using a High capacity cDNA preparation kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using Step One quantitative PCR equipment using

Power CYBR PCR kit (Applied Biosystems). *Gapdh* was used as an internal standard. PCR primer sequences were as follows: *cd11b*-forward: 5′-GCC GGT GAA ATA TGC TGT CT-3′, *cd11b*-reverse: 5′-TTC TCT GAG GCC GTG AAG TT-3′, *p67*^{phox}-forward: 5′-CGG ACA AGA AGG ACT GGA AG-3′, *p67*^{phox}-reverse: 5′-GCA TCC CTC GTT GGA AGT AA-3′, *ho-1*-forward: 5′-CTT CTT CAC CTT CCC CAA CA-3′, *ho-1*-reverse: 5′-AGC TCC TGC AAC TCC TCA AA-3′, *gapdh*-forward: 5′-TTG GTA TCG TGG AAG GAC TC-3′ and *gapdh*-reverse: 5′-TAG AGG CAG GGA TGA TGT TC-3′.

Oxidative stress measurement using APF

HL 60 cells were added with 5 μM APF and incubated for 15 min at 37°C. Then H_2O_2 (15 μM) or Tau-Cl (200 μM) was added and incubated for 30 min. Cells were collected, washed once with D-PBS(–) and resuspended in D-PBS(–) containing 0.5 μg/ml PI. The stained cells were analysed by a flow cytometer. Data were collected from PI(–) cells.

Methionine sulphoxide reductase activity

The effect of DMSO on MSR activity was studied as described previously with minor modification [22]. The reaction mixture consisted of 20 mM Tris-HCl (pH 7.4), 20 mM DTT, 1 mM MetO and 1 mg protein/ml crude MSR preparation with or without 1.2% (v/v) DMSO. The incubation was carried out in a water bath at 37°C for 3 h. The reaction was stopped by addition of sulphosalicylic acid (1%(w/v)), then centrifuged and the produced methionine in the supernatant was determined using an Hitachi L-8500 amino acid analyser at Okayama University Central Laboratory. The crude MSR preparation was prepared from differentiated HL 60 cells because they have higher MSR activity than undifferentiated cells [23]. Briefly, HL60 cells were cultured with 1 μM ATRA for 3 days, then the cells were collected and disrupted in a lysis buffer (20 mM sodium phosphate buffer (pH 7.4), 40 mM β-glycerophosphate, 20 mM NaF, 1 mM Na_3VO_4, 20 mM p-nitrophenyl phosphate, 0.1% (v/v) Nonidet-P40 and protease inhibitor cocktail (Complete mini, Roche)). Then the lysate was passed through a Sephadex G25 column which had been equilibrated and eluted with D-PBS(–) and the high molecular weight fraction was collected. The protein concentration was measured by Coomassie Plus protein assay kit (Thermo Fisher Scientific, Rockford, IL), which was typically ~ 2 mg/ml.

Amino acid analysis

Whole-cell lysate was prepared using the lysis buffer as described above and the high molecular weight fraction was separated with a Sephadex G25 column, which has been equilibrated and eluted with D-PBS(–). About 100 μg of the protein was alkaline hydrolysed as described previously [24] and the amino acid composition was analysed with an Hitachi L-8500 amino acid analyser (Tokyo, Japan).

Statistics

Group means were compared using Student's *t*-test. Analysis of variance was also used for multiple comparison using Statcel QC software (OMS publishing Inc., Saitama, Japan). The *p*-values less than 0.05 were considered to be significantly different.

Results

Oxidative stress induces HL 60 cell differentiation

Figures 1A and B show dose–response curves of CD11b expression upon DMSO and ATRA stimulation, respectively. As 1.8% DMSO was cytotoxic, especially when combined with oxidants, the following experiments were performed with 1.2% DMSO. As for ATRA concentration, 0.5 μM was chosen because this was the lowest concentration that induced a comparable degree of CD11b expression as 1.2% DMSO.

Oxidative stress increased CD11b expression (Figures 1C and D). DMSO at 1.2% significantly increased CD11b expression, which was further elevated when H_2O_2 (15 μM) or Tau-Cl (200 μM) was added at the same time of DMSO addition. Similarly, ATRA-induced CD11b expression was also increased by H_2O_2 and Tau-Cl. Moreover, oxidants alone also induced a significant CD11b expression. A synergistic effect was observed between DMSO/ATRA and H_2O_2 by analysis of variance. To confirm that the observed CD11b increase was not merely an artifact from early apoptotic cells, annexin V positive cells were also measured at 48 h. Annexin V positive cells were less than 4% of PI(–) cells in all samples, which showed that almost all CD11b(+), PI(–) cells were viable. CD14 did not increase in any of these samples (data not shown). Cell viability was more than 80% except for DMSO + Tau-Cl and ATRA + Tau-Cl samples, in which the cell viability was ~ 70%. Higher doses of oxidants resulted in substantial increase in cell death (data not shown).

Superoxide production after PMA stimulation was also studied in these cells. As expected, H_2O_2 and Tau-Cl treatment resulted in an increase in the cells that produced superoxide, as measured by NBT reduction (Figure 1E).

Cell differentiation usually accompanies growth inhibition. Thus, cell proliferation was measured by the cell counts. After 48 h of cell culture, H_2O_2 and Tau-Cl-treated samples showed significantly lower cell density (Figure 1F).

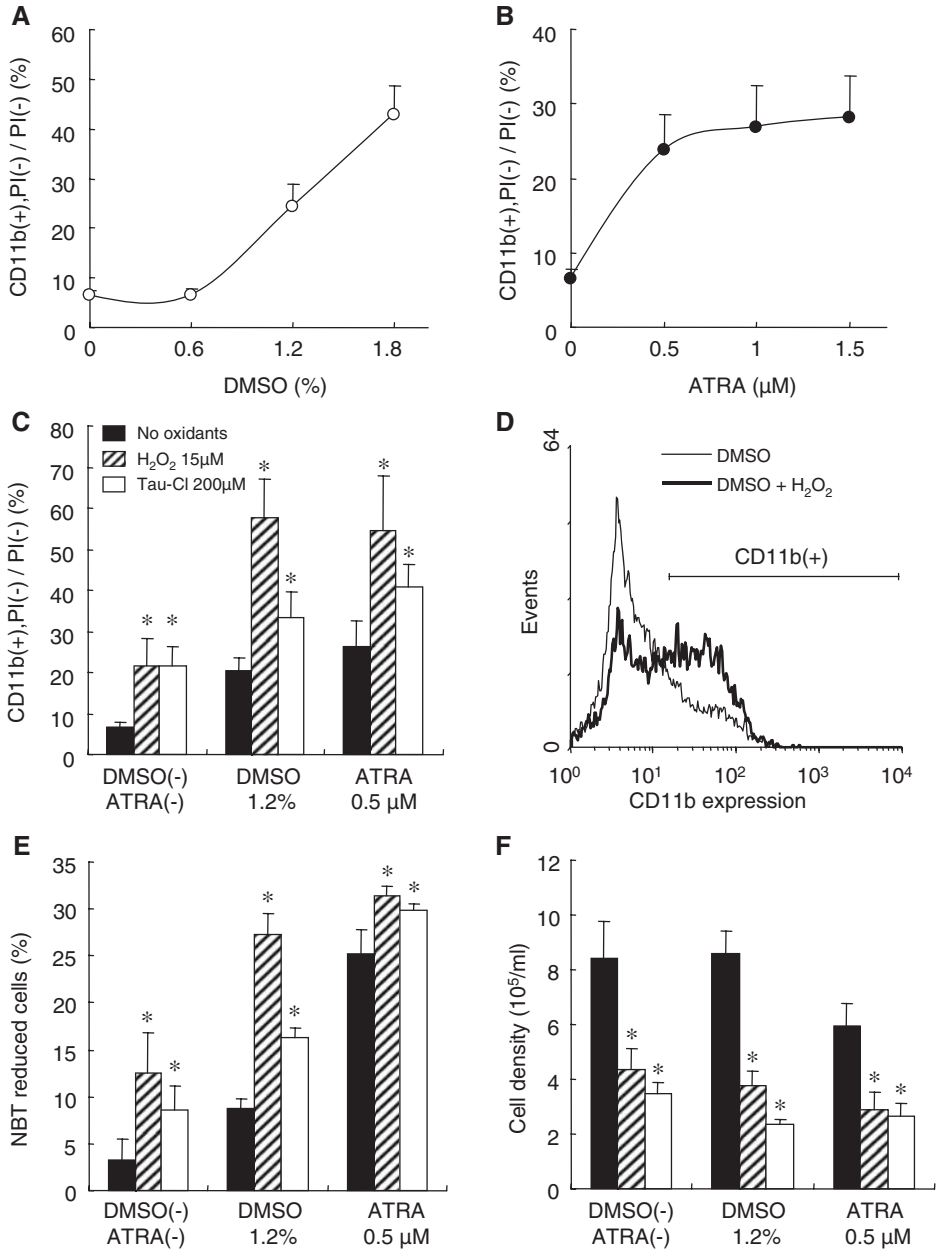

Figure 1. Oxidative stress induces HL60 cell differentiation. (A, B) HL60 cells (2×10^5 cells/ml) were added with the indicated concentrations of DMSO and ATRA and cultured for 48 h. CD11b expression was measured by flow cytometry using anti-CD11b antibody. The ratio of CD11b(+), PI(–) cells were expressed as a percentage of PI(–) cells. (C–F) HL60 cells with or without 1.2% DMSO or 0.5 μM ATRA were added with 15 μM H_2O_2 or 200 μM Tau-Cl and cultured for 48 h. (C) CD11b expression was measured as described above. (D) Typical histogram of CD11b expression from the panel (C) results. Thin line: DMSO 1.2%, no oxidant treated cells, thick line: DMSO 1.2%, H_2O_2 15 μM treated cells. (E) Superoxide production was measured by NBT reduction assay. Cells were collected and incubated with 0.33 μM of PMA and 0.5 mg/ml of NBT for 15 min at 37°C. Then the cells were smeared and observed under a light microscope. Blue formazan positive cells were expressed as a percentage of total intact cells. (F) Growth inhibition with cell differentiation. Cell numbers were counted at 48 h of culture. Values are mean ± SD from at least three independent experiments. Asterisks indicate significant difference from the corresponding 'no oxidants' samples ($p < 0.05$).

Oxidative stress increases gene expression of cd11b and p67^{phox}

As the protein levels of CD11b and NADPH oxidase activity were elevated by oxidative stress, we next studied the mRNA levels of *cd11b* and *p67^{phox}*, a cytosolic component of NADPH oxidase. As shown in Figures 2A and B, oxidative stress increased the mRNA levels of both *cd11b* and *p67^{phox}*. Notably, the increase in *cd11b* mRNA levels parallelled that of CD11b protein levels (Figures 1C and 2A), which suggested that oxidative stress increased CD11b at the transcription level. The increase in *p67^{phox}* was also consistent with that of NBT reduced cells (Figures 1E and 2B).

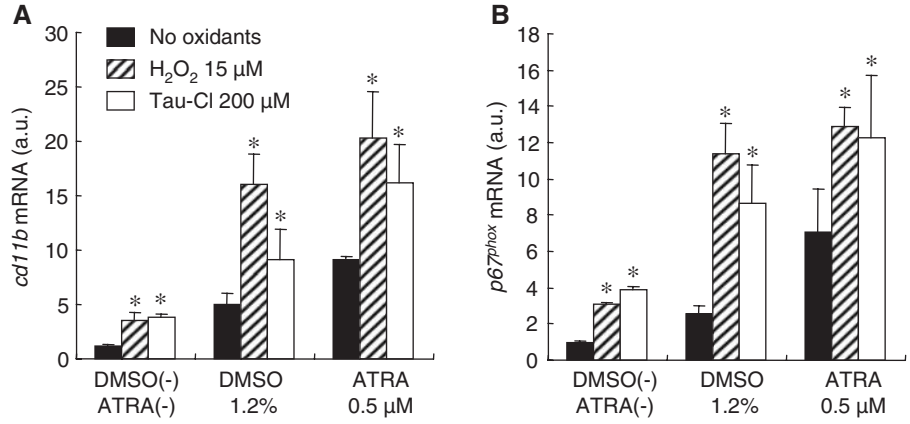

Figure 2. Increase in differentiation-associated mRNA expression by oxidative stress. Cells were treated as described in Figure 1(C) legend. Total RNA was extracted from the cells, cDNA was prepared using random primer and the gene expression was measured by quantitative PCR. *Gapdh* was used as an internal standard and the mRNA expression was expressed as fold increase compared with DMSO(–), ATRA(–), oxidant(–) samples. Values are mean ± SD from at least three independent experiments. Asterisks indicate significant increase from the corresponding 'no oxidants' samples ($p < 0.05$).

Reducing chemicals suppress cell differentiation

If the oxidative stress is involved in cell differentiation, reducing chemicals may have opposite effects. Figure 3 showed that HL 60 differentiation by DMSO and ATRA were inhibited significantly by DTT, BAL and NAC. Dithiol compounds (DTT and BAL) were effective at lower concentration than mono-thiol compound NAC. These results suggest that thiol compounds, particularly dithiol compounds, were effective in the suppression of differentiation. Addition of the reductants for the last 10 min resulted in no significant change in CD11b expression, which indicated that the reductants did not interfere with the CD11b detection (data not shown).

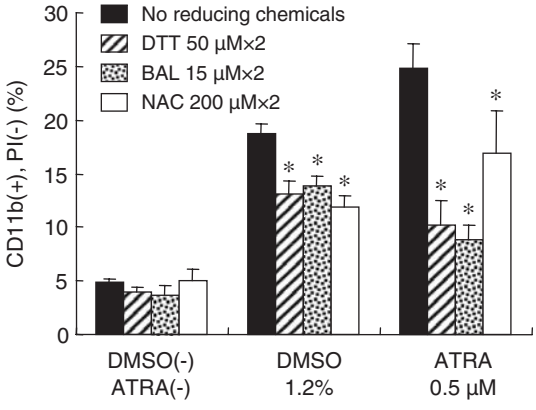

Figure 3. Reducing chemicals inhibit HL 60 differentiation. HL60 cells (2×10^5 cells/ml) with or without 1.2% DMSO or 0.5 μM ATRA were added with 50 μM DTT, 15 μM BAL (2,3 dimercapto-1-propanol) or 200 μM NAC and cultured for 24 h. Then the same dose of DTT, BAL or NAC was added again and the cells were cultured for an additional 24 h. At 48 h, the cells were collected and CD11b expression was measured by a flow cytometer. The ratio of CD11b(+), PI(–) cells were expressed as a percentage of PI(–) cells. Values are mean ± SD from at least three independent experiments. Asterisks indicate significant decrease from the corresponding 'no reducing chemicals' samples ($p < 0.05$).

DMSO works as an oxidative stress

As the structure of DMSO and the side chain of MetO are similar (Figure 4A), DMSO can be a substrate for MSR [25]. Thus, the effect of DMSO on the MSR activity was studied. In the complete reaction system (20 mM Tris-HCl, 1 mM MetO, 20 mM DTT and 1 mg protein/ml crude MSR preparation), MetO was reduced to Met using DTT as reducing substrate (Figure 4B). When DTT was omitted, Met production was mostly inhibited. Even in the presence of DTT, 1.2%(v/v) DMSO also inhibited Met production almost completely (Figure 4B). As 1.2% (v/v) DMSO was equal to ~ 169 mM, the result suggested that DMSO inhibited MSR competitively.

In addition, DMSO enhanced cell death by oxidative stress. HL 60 cells underwent cell death by NH_2Cl [26], a physiological oxidant produced by neutrophils. When the cells were supplemented with 1.2% DMSO, the cell death increased significantly (Figure 4C).

Moreover, DMSO at 1.2% concentration induced *ho-1* expression (Figure 4D). DMSO at 1.2% induced *ho-1* expression as well as cell differentiation (Figures 3 and 4D) and 0.6% DMSO did not induce significant *ho-1* expression, where CD11b expression was negligible (Figure 1A). It was noted that *ho-1* induction was neither a pre-requisite nor a result of cell differentiation, because ATRA induced cell differentiation without *ho-1* induction.

Oxidative stress response precedes the induction of differentiation-associated genes

Cellular oxidative stress was measured by two methods, i.e. APF and *ho-1* expression. APF generates

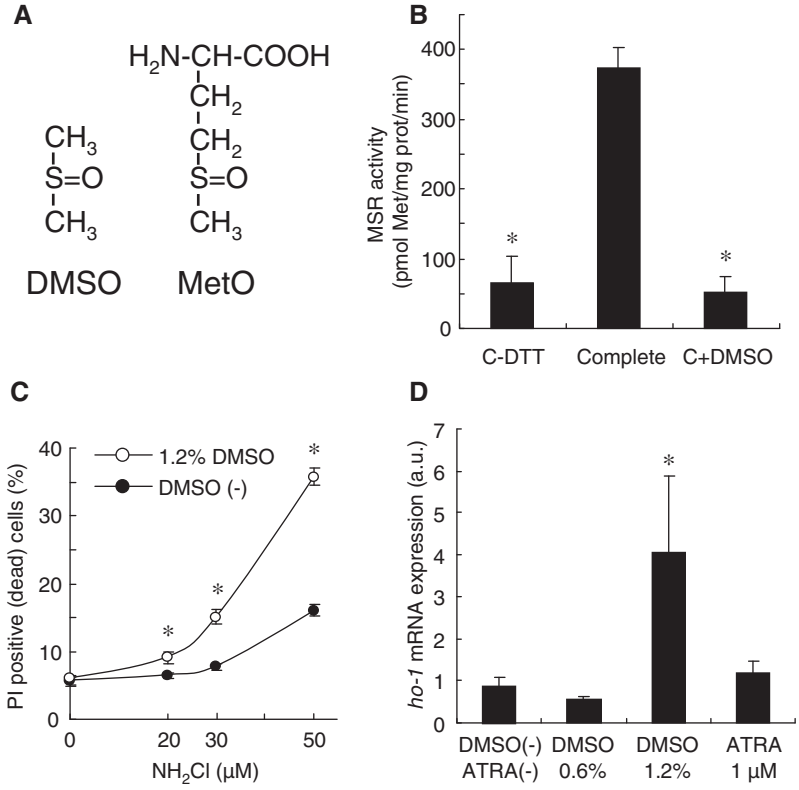

Figure 4. DMSO works as oxidative stress. (A) The structure of DMSO and MetO. (B) DMSO inhibited MSR activity. The 'Complete' system contained 20 mM Tris-HCl pH 7.4, 20 mM DTT, 1 mM MetO and 1 mg protein/ml crude MSR preparation. C-DTT: DTT was omitted from the complete system. C+DMSO: DMSO (1.2%) was added to the complete system. The mixture was incubated for 3 h at 37°C and the generated methionine was measured by an amino acid analyser. Asterisks indicate significant decrease from 'complete' samples ($p < 0.05$). (C) DMSO augments oxidative stress-induced cell death. HL60 cells with or without 1.2% DMSO were added with the indicated concentrations of NH_2Cl and cultured for 24 h. The cells were collected, stained with PI and analysed by a flow cytometer. Asterisks indicate a significant increase from the corresponding 'DMSO(–)' samples ($p < 0.05$). (D) DMSO at 1.2% induces *ho-1* mRNA expression in HL60 cells. HL60 cells were incubated with the indicated chemicals for 48 h, and the mRNA expression was studied by quantitative PCR. Asterisks indicate significant increase from 'DMSO(–), ATRA(–)' samples ($p < 0.05$). Values are mean ± SD from at least three independent experiments.

fluorescein in the direct reaction with oxidants [27]. When HL 60 cells were pre-incubated with APF and then incubated with H_2O_2 and Tau-Cl for 30 min, cellular fluorescence increased significantly (Figure 5A). To study further the cause–effect relation of oxidative stress and cell differentiation, the time-courses of *ho-1* and *cd11b* mRNA expression were compared. Figure 5B shows that Tau-Cl and H_2O_2 induced a rapid elevation of *ho-1* mRNA at 6 h. In contrast, *cd11b* mRNA showed no significant change at 6 h, but increased significantly at 48 h (Figure 5C). The results indicated that oxidative stress preceded the expression of differentiation-associated genes.

Involvement of Met and dithiol oxidation in cell differentiation

If DMSO and MetO compete for MSR, the consequent metabolic alteration may be MetO accumulation and/or enhanced oxidation of Trx, a physiological MSR substrate (Figure 6A). Thus, we studied if Met levels decrease as a result of MetO accumulation. Consistent with this assumption, Met levels showed a slight but significant decrease after DMSO addition. Moreover, DMSO and Tau-Cl synergistically decreased Met levels (Figure 6B). It was difficult to quantify MetO levels directly, because MetO peaks overlapped Ser and Thr peaks.

Thioredoxin can be oxidized effectively with vicinal dithiol-reactive oxidant, PAO. Interestingly, PAO at 50 nM also increased CD11b expression (Figure 6C). It was noted that effective PAO concentration was at least two orders of magnitude lower than that of oxidants, which suggested that vicinal dithiol has a critical role in cell differentiation.

Discussion

In this experiment, H_2O_2 and Tau-Cl increased CD11b expression, NBT reduction activity and inhibited cell proliferation in HL 60 cells. The differentiated cells showed nuclear segmentation, and they did not express CD14 (data not shown). These results indicated that oxidative stress enhanced granulocytic

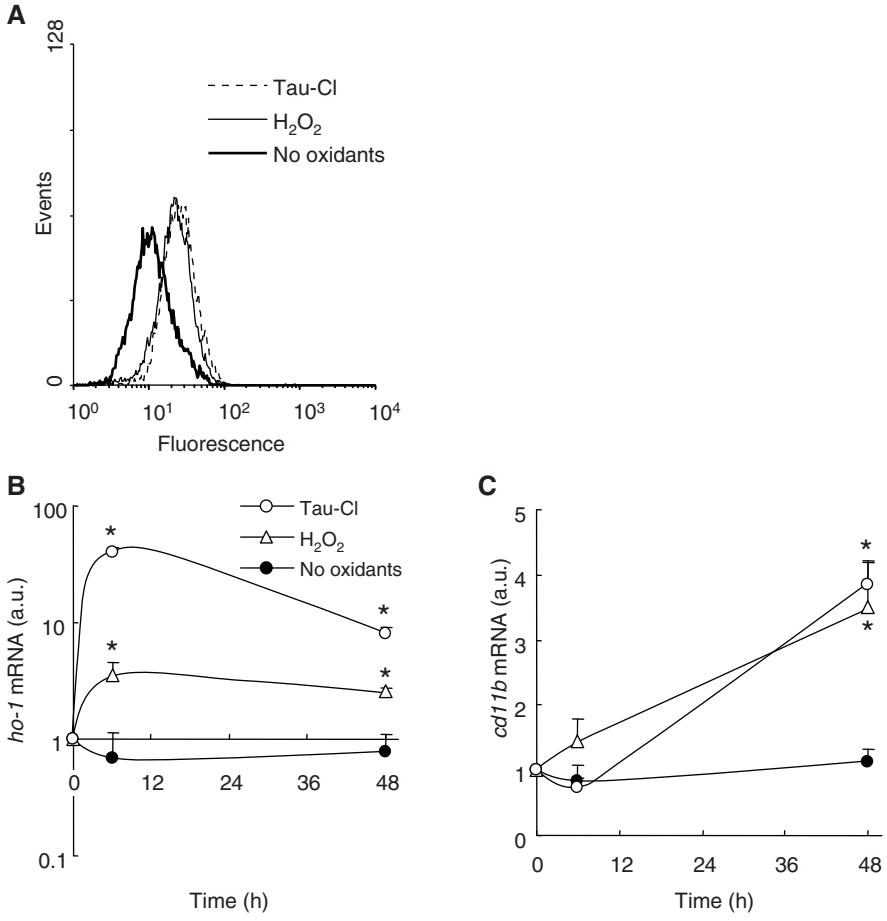

Figure 5. *Ho-1* expression precedes *cd11b* expression. (A) HL60 cells (2×10^5 cells/ml) were pre-incubated with 5 μM APF for 15 min and then H_2O_2 (15 μM) or Tau-Cl (200 μM) were added and the incubation was continued for 30 min. Cellular fluorescence was measured by a flow cytometer. Thick line: no oxidants, thin line: H_2O_2, broken line: Tau-Cl. (B, C) HL60 cells were added with 15 μM H_2O_2 or 200 μM Tau-Cl and cultured for 6 and 48 h. Cells were collected and the mRNA expression was measured by quantitative PCR. *Gapdh* was used as an internal standard and the mRNA expression was expressed as fold increase compared with 'no oxidants, time = 0 h' samples. Values are mean ± SD from at least three independent experiments. Asterisks indicate significant increase from the corresponding 'no oxidants' samples ($p < 0.05$).

differentiation. Although the degree of cell differentiation was modest, it was probably because the measurements were done at 48 h of differentiation, as longer incubation over-crowded the control cells. Tau-Cl is a mild oxidant and preferentially reacts with thiols and thioethers [28]. Thus, Cys and Met residues are likely candidates for oxidative modification. In many cells, reduced glutathione exists at millimolar concentration in the cytoplasm, which is a major defence against oxidative stress. Nevertheless, increasing evidence indicates that thiol groups of various proteins, such as protein kinases, protein phosphatases and transcription factors, are oxidatively modified under physiological as well as pathological settings, and that cellular responses to oxidative stress are based on these protein modifications [29]. Oxidized protein thiols can be reduced by Trx or dithiol compounds [30]. Consistently, dithiol compounds such as DTT and BAL were effective at lower concentrations than NAC in our experiment, which suggests the involvement of protein thiol oxidation in cell differentiation. Physiologically, Trx is important for protein disulphide reduction [31,32]. Thus, the lack of reduced Trx may augment oxidant effects by a delayed reduction of protein disulphide. This mechanism may also be involved in the effects of DMSO. As DMSO can be a substrate for MSR, Trx oxidation is expected to increase, which delays protein disulphide reduction. The importance of vicinal dithiol oxidation is also supported by the finding that PAO also induced cell differentiation at 50 nM, which was two and three orders of magnitude lower concentration than H_2O_2 and Tau-Cl, respectively. Although PAO preferentially reacts with vicinal dithiols [33], the finding does not rule out the possibility that Met oxidation plays some role in oxidative stress-induced cell differentiation, because MetO is reduced back to Met by MSR using Trx [19], and PAO is likely to inhibit this reaction by oxidizing thioredoxin [34]. Collectively, oxidation of critical Cys or Met residues may be responsible for cell differentiation.

Oxidative stress is not a pre-requisite for cell differentiation, because ATRA effectively induced HL60

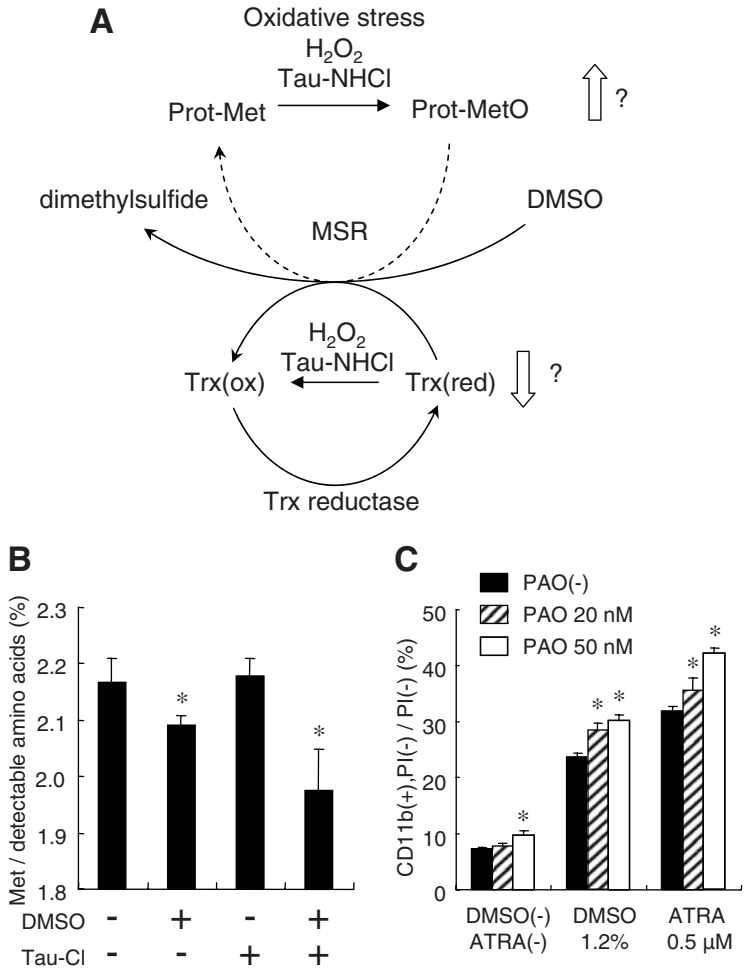

Figure 6. Involvement of Met and dithiol oxidation in cell differentiation. (A) Possible metabolic alteration by DMSO and/or oxidative stress. DMSO is likely to inhibit Prot-MetO reduction (broken line) and enhance Trx oxidation. (B) HL60 cells (2×10^5 cells/ml) were added with 1.2% DMSO and/or 200 μM Tau-Cl and incubated for 3 h. Then the cell lysate was prepared and the high molecular weight fraction was separated using a Sephadex G-25 column. The amino acid composition of the cell lysate protein was determined after alkaline hydrolysis, and the Met contents were shown as a percentage of total detectable amino acids. Values are mean ± SD from at least three independent experiments. Asterisks indicate significant decrease from the DMSO(–), Tau-Cl(–) samples ($p < 0.05$). (C) HL60 cells (2×10^5cells/ml) with or without 1.2% DMSO or 0.5 μM ATRA were added with 20 and 50 nM PAO and cultured for 48 h. CD11b expression was measured by flow cytometry using anti-CD11b antibody. The ratio of CD11b(+), PI(–) cells were expressed as a percentage of PI(–) cells. Values are mean ± SD from at least three independent experiments. Asterisks indicate significant increase from the corresponding 'PAO(–)' samples ($p < 0.05$).

differentiation without enhancing *ho-1* expression. Nevertheless, oxidative stress enhanced ATRA-induced differentiation, whereas reducing chemicals inhibited the differentiation. These results suggest that retinoic acid and oxidative stress may work independently in the HL60 differentiation. It is also important that reducing reagents, including NAC, inhibited ATRA-induced HL 60 differentiation. As NAC is widely used clinically, the combination of ATRA and NAC may diminish APL differentiation by ATRA.

It is another interesting finding that oxidative stress is involved in DMSO-induced HL60 differentiation. It has been well recognized that DMSO induces differentiation in various cell lines, such as K562, murine Friend leukaemia and HL60. However, the mechanism was not fully understood. Although DMSO is a hydroxyl radical scavenger [35] and works as an antioxidant in some conditions, it also works as oxidative stress to the cells. First, DMSO inhibited the activity of methionine sulphoxide reductase [25]. Indeed, DMSO induced a slight but significant decrease in protein Met (Figure 6B), probably by a delayed reduction of protein MetO (Figure 6A, broken line). It should be noted that oxidation of another substrate of MSR, Trx, is expected to increase. Secondly, DMSO enhanced apoptosis induced by NH_2Cl, one of the physiological oxidants produced by neutrophils. As NH_2Cl preferentially oxidizes Cys and Met residues [36], and as the protein oxidation appears to be the cause of various effects of NH_2Cl [17,37], the enhanced apoptosis was

probably related to MSR inhibition. Thirdly, *ho-1* mRNA expression was increased by 1.2% DMSO treatment. *Ho-1* expression is regulated by Nrf2 transcription factor, which is activated by oxidative stress [13]. It is interesting to note that 0.6% DMSO, a concentration that showed minimal HL60 differentiation, failed to induce significant *ho-1* over-expression. This result also supports that oxidative stress is involved in DMSO-induced HL60 differentiation.

ATRA-induced differentiation markedly improved the outcome of APL. Nevertheless, as ATRA is not effective to all types of leukaemia, we need to know more about leukaemic cell differentiation. In this respect, DMSO is interesting because it also induces differentiation in several leukaemic cell lines and it enhances oxidative stress. It is likely that oxidative stress stimulates granulocytic differentiation in leukaemic cells and that cellular differentiation can be modified by redox control.

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