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Original Paper

Mitochondrial Activity and Unfolded Protein Response are Required for Neutrophil Differentiation

Ayako Tanimura[d,a] Keiko Miyoshi[a] Taigo Horiguchi[a] Hiroko Hagita[a]
Koichi Fujisawa[b,c] Takafumi Noma[a]

[a]Department of Molecular Biology, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, [b]Center of Research and Education for Regenerative Medicine, Yamaguchi University Graduate School of Medicine, Ube, [c]Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Ube, [d]Department of Food and Health Sciences, Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, Kumamoto, Japan

Key Words
ATP • ER stress • HL-60 • Mitochondria • Myelocytic differentiation • Unfolded protein response

Abstract
Background/Aims: Endoplasmic reticulum (ER) stress and unfolded protein response (UPR) are involved in hematopoietic differentiation. However, the mechanistic linkage between ER stress/UPR and hematopoietic differentiation remains unclear. ***Methods:*** We used bipotent HL-60 cells as an *in vitro* hematopoietic differentiation system to investigate the role of ER stress and UPR activity in neutrophil and macrophage differentiation. ***Results:*** The *in vitro* differentiation analysis revealed that ER stress decreased during both neutrophil and macrophage differentiations, and the activities of PERK and ATF6 were decreased and that of IRE1α was increased during neutrophil differentiation in a stage-specific manner. By contrast, the activities of ATF6 and ATF4 decreased during macrophage differentiation. When the cells were treated with oligomycin, the expression of CD11b, a myelocytic differentiation marker, and morphological differentiation were suppressed, and XBP-1 activation was inhibited during neutrophil differentiation, whereas CD11b expression was maintained, and morphological differentiation was not obviously affected during macrophage differentiation. ***Conclusion:*** In this study, we demonstrated that neutrophil differentiation is regulated by ER stress/UPR that is supported by mitochondrial ATP supply, in which IRE1α-XBP1 activation is essential. Our findings provide the evidence that mitochondrial energy metabolism may play a critical role in neutrophil differentiation.

Takafumi Noma

Dept. of Molecular Biology, Institute of Biomedical Sciences, Tokushima University Graduate School, 3-18-15 Kuramoto, Tokushima 770-8504 (Japan)
Tel. +81-88-633-7326, Fax +81-88-633-7326, E-Mail ntaka@tokushima-u.ac.jp

Introduction

Protein synthesis occurs in ribosomes present in cells. Following production, these proteins are transported into the endoplasmic reticulum (ER) where they are folded into their tertiary structure. When misfolded proteins are accumulated in the ER, it causes an overload of mutant or denatured proteins. This event can lead to cell damage or apoptosis, which is known as "ER stress." However, cells can adapt to ER stress via the unfolded protein response (UPR), which consists of three signaling pathways via ER-stress sensors, IRE1α, PERK, and ATF6. Once UPR is triggered by ER stress, misfolded proteins are eliminated by three mechanisms, such as producing chaperones to promote protein folding, inhibiting mRNA translation, and inducing unfolded protein degradation (ER-associated degradation; ERAD) [1, 2].

On the other hand, ER stress and UPR are involved in several cellular differentiations [3]. For example, during adipocyte and B-cell differentiation, ER stress increased and *Xbp-1* splicing was induced through IRE1α activation [4]. When XBP1 activation was inhibited by blocking the supply of mitochondrial ATP through the knockdown of adenylate kinase 2 (AK2), an adenine nucleotide converting enzyme in the mitochondrial intermembrane space, adipocyte and B-cell differentiation was impaired [4]. Additionally, *Xbp-1* knockout in mice shows impaired eosinophil differentiation [5]. However, the molecular mechanism linking ER stress/UPR, ATP supply, and cell differentiation remains unclear.

Here we studied the cell-specific roles of ER stress and UPR activity during neutrophil and macrophage differentiation with the *in vitro* hematopoietic differentiation system using the bipotent myeloid cell line HL-60.

Materials and Methods

Cell culture and differentiations

HL-60 human promyelocytic leukemia cells were provided by the RIKEN BioResource Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan [6, 7]. HL-60 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS). Neutrophil differentiation was induced by treating HL-60 cells (2.5×10^5 cells/ml) with 10 μM all-trans retinoic acid (ATRA) (Sigma, St. Louis, MO) in a flask [8]. Macrophage differentiation was induced by treating HL-60 cells (1×10^5 cells/ml) with 20 nM phorbol myristate acetate (PMA) (Sigma) in a 6-well plate [9].

Reagents

To reduce ER stress induced by misfolded proteins, HL-60 cells (2.5×10^5 cells/ml) were treated with 0.67, 2, 6 mM of 4-phenylbutyric acid (4-PBA) (Sigma), a chemical chaperone, in a flask for 4 days. For UPR inhibition, we used the following compounds: 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) (02068-64; NACALAI TESQUE, Kyoto, Japan) as ATF6 inhibitor, GSK2606414 (G7345; LKT laboratories, St. Paul, MN) as PERK inhibitor, and 8-formyl-4-methylumbelliferone (4μ8C) (4479/10; R&D Systems, Minneapolis, MN) as IRE1α-XBP1 inhibitor. To inhibit mitochondrial ATP production, we used oligomycin (#11341; Cayman Chemical, Ann Arbor, MI). As a positive control, we applied 0.6 μg/ml of tunicamycin to the HL-60 culture for 8 h to induce ER stress.

Western blot

Western blot analyses were performed as previously described [10]. For electrophoresis, we used 20 μg of sample protein. After subsequently transferring the proteins to PVDF membranes and applying a nonspecific epitope blocking using 5% skimmed milk, the following antibodies were applied for 1 h or overnight: human integrin alpha M/CD11b (238439) (R&D systems), anti-XBP1 antibody (ab37152; Abcam, Cambridge, MA), CREB-2 antibody (C-20) (cs-200; Santa cruz, Santa Cruz, CA) as ATF4 antibody, anti-ATF6 antibody (ab37149; Abcam, and sc-166659; Santa Cruz), anti-caspase-3 antibody (9662S; Cell Signaling Technology, Danvers, MA) and monoclonal anti-β-actin Clone AC-15 (Sigma) as a loading control. ECL anti-rabbit IgG horseradish peroxidase-linked whole antibody and anti-mouse IgG (GE Healthcare, Munich,

Germany) were used as secondary antibodies. Immunodetection signals were identified using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) and exposed to Fuji medical X-ray film (Fujifilm, Tokyo, Japan).

Wright-Giemsa staining

Cells were analyzed by staining with Wright-Giemsa solution (Muto Pure Chemicals, Tokyo, Japan) according to the manufacturer's protocol. Briefly, cells were smeared on glass slides manually or prepared by CytoSpin4 Cytocentrifuge (Thermo Fisher Scientific, Waltham, MA). Then, they were dried and stained with Wright-Giemsa solution for 2 min. After diluting M/150 phosphate buffer, cells were stained for 8 min and were then washed.

NBT assay

Cells (6×10^5 cells) were incubated with NBT solution (Muto Pure Chemicals, Tokyo, Japan) for 30 min at 37°C. Then, the cells were suspended in PBS, and NBT-positive cells (about 100 cells) were counted, and calculated the ratio of NBT positive cells to the total number of cells.

Apoptosis analysis

Apoptotic cells were counted by ~ 55 fields in each slide with Wright-Giemsa staining, and the apoptotic rate was calculated as the percentage of apoptotic cells to total cells.

Measurement of intracellular ATP amount

ATP content was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's instruction. Briefly, 100 µl of the cell culture was transferred into a black 96-well dish, and the same amount of CellTiter-Glo reagent was added into the wells. After shaking the plate for 30 s and leaving it to rest for 10 min, the amount of ATP in the well was measured using Tristar LB 941 luminometer (Berthold Japan, Tokyo, Japan). Each value was normalized by total protein amount.

Statistics

Each analysis was performed in triplicate, and all experiments were conducted under the same experimental conditions. We used the Image J software (http://rsb.info.nih.gov/ij/) to quantify the western blot data. The data were normalized by β-actin content. Graphs show means ± SD. Student's *t*-test was performed in Microsoft Excel 2010 and a *P*-value of <0.05 was considered as statistically significant.

Results

ER stress levels during hematopoietic differentiation of HL-60 cells

HL-60 cells can differentiate into neutrophils or macrophages by ATRA or PMA, respectively. To explore the role of ER-stress and UPR in the myeloid differentiation of HL-60 cells, we first examined the expression of BiP, an ER stress marker, using western blot analysis. BiP expression was elevated in non-treated HL-60 cells and reduced during both ATRA-induced neutrophil differentiation and PMA-induced macrophage differentiation (Fig. 1). In neutrophil differentiation, BiP expression on day 4 remained at approximately 10% of that on day 1. During macrophage differentiation, we also observed reduction in BiP expression to that on day 1. We confirmed that neutrophil and macrophage differentiation is completed at days 4 and 1, respectively, based on morphological changes and marker expressions as shown previously [11]. Thus, we found the reduction in ER stress marker expression during myeloid differentiation in our system. Then we evaluated the effect of ER stress on neutrophil differentiation in HL-60 cells. HL-60 cells were treated with 4-PBA, a chemical chaperone, which reduces ER stress [12], and assessed ER stress level by BiP and differentiation by CD11b differentiation marker. 4-PBA treatment reduced ER stress marker expression dose-dependently, and concomitantly both up-regulation of CD11b expression and nuclear morphological changes were observed with 2mM of 4-PBA (Fig. 1c, d). These results suggested that neutrophil differentiation is controlled by ER stress level.

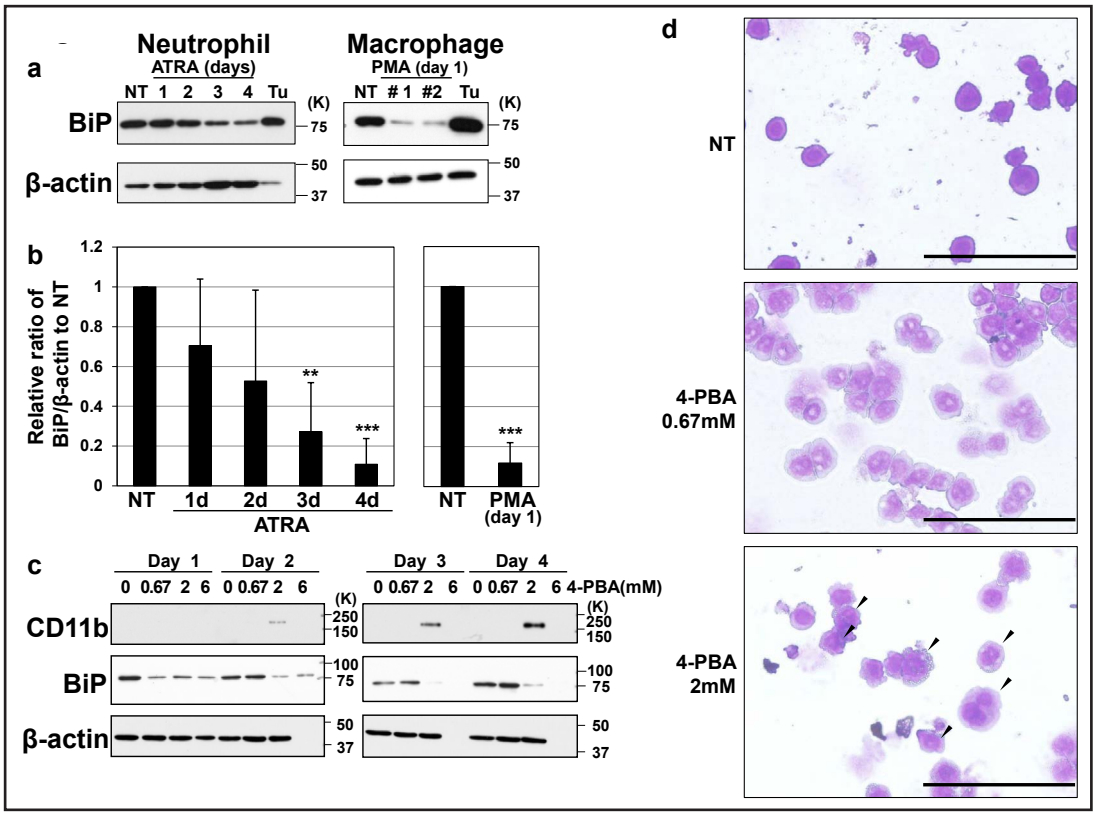


Fig. 1. Expression level of the ER-stress marker BiP in HL-60 cells following ATRA and PMA treatments. (a) Dynamic change in ER stress during neutrophil and macrophage differentiation. BiP expression was detected as a marker of ER stress using BiP antibody. #1 and #2 represent different samples. β-actin is shown as a loading control. (b) Relative BiP expression level. Data of western blot analysis in (a) are represented using graphs. The relative ratio is calculated using the value of non-treated HL-60 cells (NT) as 1. Tu indicates the samples of HL-60 cells treated with tunicamycin (0.6 μg/ml, 8 h) as a positive control. N = 3. ** P<0.01, *** P<0.001. (c) HL-60 cells were treated with 4-PBA at the doses indicated in the Fig. CD11b expression was detected as a marker of myeloid differentiation. Cells treated with 6 mM of 4-PBA for 2, 3, and 4 days were dead; thus, β-actin signals were not detected. (d) Wright-Giemsa staining was performed with cells treated with or without 4-PBA for 4 days. Cells treated with 6 mM of 4-PBA were not shown because of cell death. Arrowheads, segmented neutrophils.

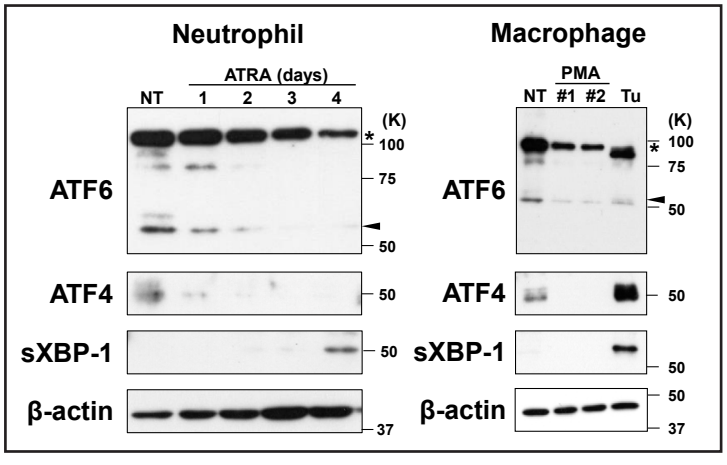


Fig. 2. UPR activity during neutrophil and macrophage differentiation in HL-60 cells. Activation of UPR pathways, such as ATF6, PERK, and IRE1α, during neutrophil and macrophage differentiation was detected by western blot analysis. Cleaved ATF6 (arrowhead), ATF4, and spliced XBP-1 (sXBP-1) were the markers of the ATF6, PERK, and IRE1α-XBP1 pathways, respectively. Asterisks indicate full-length ATF6. β-actin is shown as a loading control. Tu indicates the samples of HL-60 cells treated with tunicamycin as a positive control. N = 3.

UPR activity during differentiation in HL-60 cells

Next, we further analyzed UPR activity in response to ER stress dynamics in neutrophil and macrophage differentiation. Before inducing differentiation, we found that two UPR pathways were activated by detecting cleaved ATF6 and ATF4 in HL-60 cells (Fig. 2). This finding was consistent with BiP expression in non-treated HL-60 cells (Fig. 1), suggesting that ER stress is constantly present in bipotent HL-60 cells and that UPR in response to ER stress may help to maintain cellular homeostasis and cell survival. Following ATRA treatment, the uncleaved and cleaved forms of ATF6 and ATF4 expressions decreased gradually during neutrophil differentiation and the spliced XBP1 came out at day 4 (Fig. 2). By contrast, only the uncleaved and cleaved forms of ATF6 were observed at low level following PMA treatment for macrophage differentiation.

Essential role of UPR activity in neutrophil differentiation of HL-60 cells

Because cell-type specific UPR responses were observed, we further investigated the effect of UPR inhibition on neutrophil and macrophage differentiation (Fig. 3). At first, we

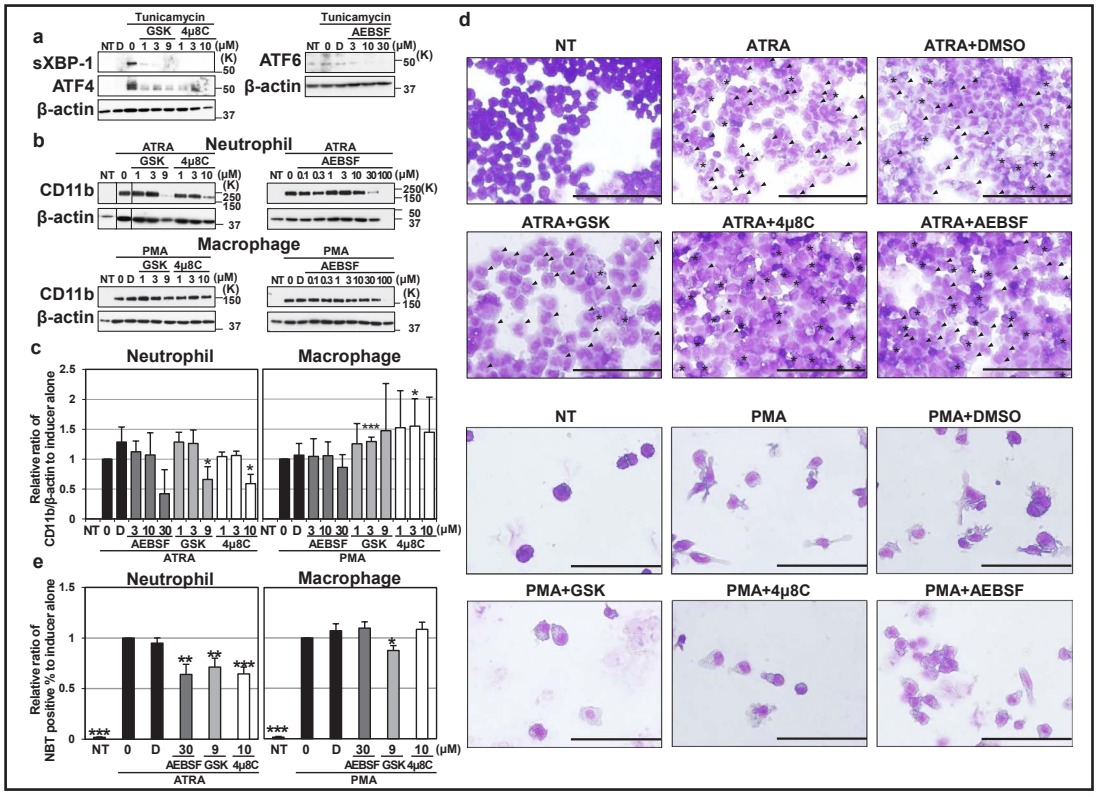


Fig. 3. Effect of UPR inhibitors on neutrophil and macrophage differentiation. (a) Effects of UPR inhibitors on the activation of each UPR pathway, which were evaluated by western blot analysis. Samples were treated with or without tunicamycin as an ER stress inducer. D indicates dimethyl sulfoxide (DMSO). NT shows non-treated HL-60 cells. (b) Effects of each UPR inhibitor on myeloid differentiation. CD11b expression was evaluated by western blot analysis at day 1 of macrophage differentiation and day 4 of neutrophil differentiation. D represents HL-60 cells treated with DMSO (vehicle) and PMA or ATRA in (b). (c) Relative CD11b expression levels with UPR inhibitors. Data of western blot analysis in (b) are represented using graphs. The relative ratio is estimated using the value of ATRA-treated or PMA-treated HL-60 cells without UPR inhibitor treatment as 1. β-actin is shown as a loading control. (d) Morphological confirmation of neutrophil and macrophage differentiation by Wright-Giemsa staining. The concentrations of inhibitors were GSK 9μM, 4μ8C 10μM, and AEBSF 30μM. Scale bar, 100 μm. Arrowheads, segmented neutrophil; asterisks, apoptotic cell. (e) Functional assessment using NBT assays. Mature differentiated macrophage- and neutrophil-like HL-60 cells are NBT positive. GSK2606414 (GSK), PERK inhibitor; 4μ8C, IRE1α inhibitor; AEBSF, ATF6 inhibitor. N = 3. * P<0.05, ** P<0.01, *** P<0.001 v.s. ATRA or PMA.

examined the effects of UPR inhibitors (GSK2606414 (GSK) for PERK, 4μ8C as IRE1α-XBP1 inhibitor, and AEBSF as ATF6) by applying them to HL-60 cells. Each inhibitor specifically inactivated the corresponding URP pathway (Fig. 3a). Next, we examined the effects of UPR inhibitors on the expression of CD11b, a myelocytic differentiation marker, during myeloid differentiation by western blot analysis (Figs. 3b and 3c). CD11b expression decreased with all UPR inhibitors during neutrophil differentiation.

Next, we checked cell morphology by Wright-Giemsa staining, and function by NBT assay. ATRA- and (ATRA+DMSO)-treated cells had segmented and band nucleus like mature neutrophil (Fig.3d). In ATRA + GSK treatment, nuclear/cytoplasmic ratio was decreased, and almost cells represented segmented or band nucleus. In ATRA + 4μ8C treatment, segmented nuclear cells were few, and many cells were myelocyte-like which had rounded nucleus, and metamyelocyte-like which had kidney-shaped nucleus. In ATRA+AEBSF treatment, some cells could differentiate into segmented- or band-neutrophils. But, there seemed to be fewer mature differentiated cells than by GSK treatment. For cellular function, all UPR inhibitors reduced NBT-positive cells to 60 - 70 % of ATRA treatment (71 % in GSK, 65 % in 4μ8C, 64 % in AEBSF).

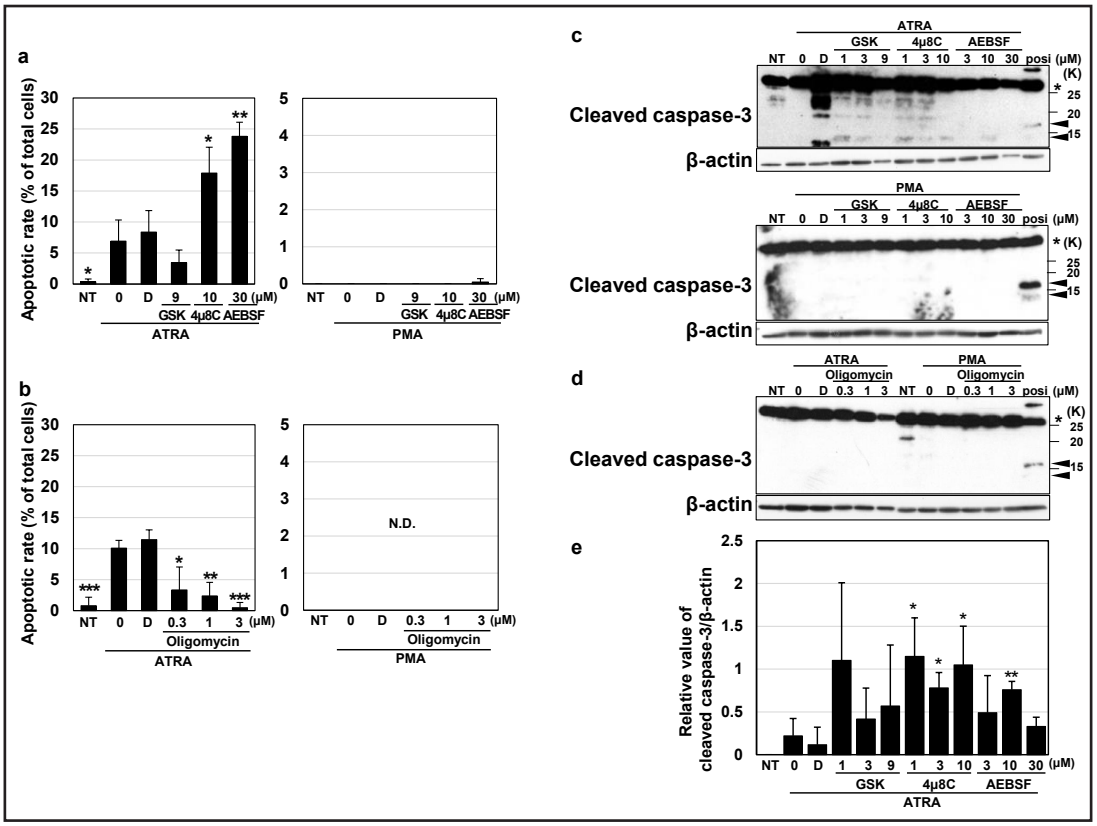


Fig. 4. Effect of UPR inhibitors and oligomycin on apoptosis in neutrophil and macrophage differentiation. Apoptosis signal was evaluated by the apoptotic rate (% of total cells) (a, b) and cleaved caspase-3 level (c - e) at day 4 of neutrophil differentiation and day 1 of macrophage differentiation. (a, b) Cells were stained with Wright-Giemsa and counted. The apoptotic rate was calculated. N.D., not detected. (c - e) Cleaved caspase-3 was detected as a marker of apoptosis. β-actin is shown as a loading control. HL-60 cells treated with 50 μM etoposide for 4 h were used as positive control. NT, non-treated HL-60 cells; D, HL-60 cells treated with DMSO (vehicle) and ATRA or PMA. Asterisks, full length caspase-3; arrowheads, cleaved Caspase-3. (e) Relative cleaved caspase-3 levels with UPR inhibitors in neutrophil differentiation. Graph is prepared with densitometric analysis of the data of western blot with ATRA and UPR inhibitors shown in (c). The concentrations of inhibitors were GSK 9 μM, 4μ8C 10 μM, AEBSF 30 μM. * P<0.05, ** P<0.01, *** P<0.001 v.s. ATRA + D.

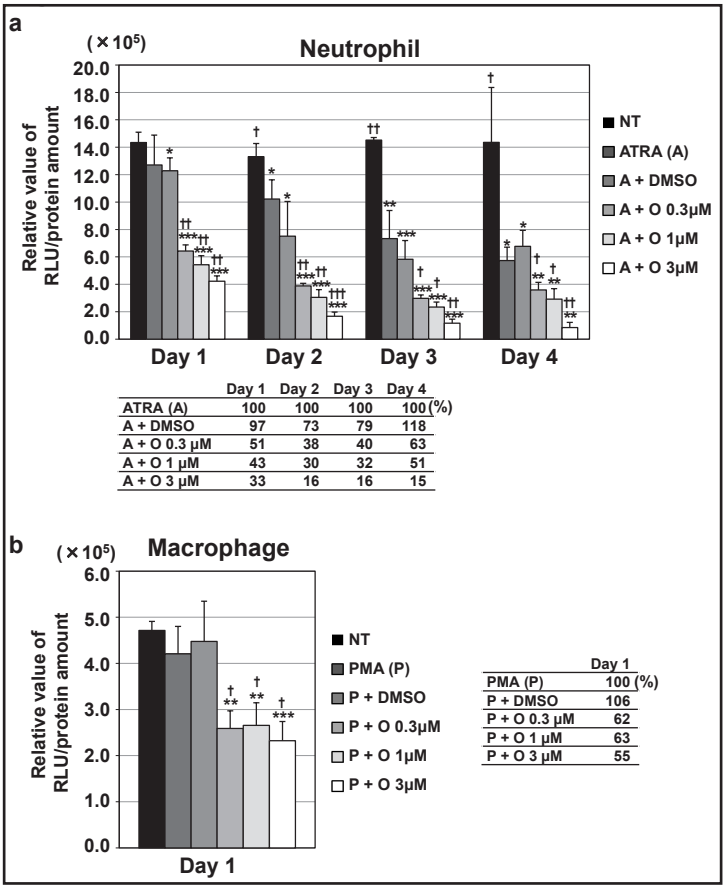
Furthermore, apoptotic cells were characterized by cell shrinkage and chromatin condensation that were found in treatments with ATRA alone or ATRA + UPR inhibitors (Fig. 3d). We found that ATRA induced apoptosis (7 % of total cells) in Fig. 4a. In UPR inhibitor treatment, 4μ8C and AEBSF enhanced apoptosis in neutrophil differentiation significantly (17.9 % and 23.8 %). In contrast, ATRA + GSK treatment seemed to decrease apoptosis ratio (3.4%) compare to ATRA + DMSO treatment (8.4 %). Consistent with these findings, the statistically significant increase of an apoptotic marker, cleaved caspase-3 was observed by 4μ8C and AEBSF treatment in neutrophil differentiation (Fig. 4c, e).

In the case of PMA treatment, no inhibitor blocked macrophage differentiation as shown by CD11b surface marker expression and morphology (Fig. 3b, c, d). In NBT assay with GSK treatment, NBT-positive cells were reduced slightly (85%) in each differentiation (Fig. 3c, e). Additionally, PMA did not induce apoptosis as shown in Fig. 4. These findings clearly demonstrated that surface marker expression, cell shape, and cellular function are regulated through UPR during neutrophil differentiation. In contrast, UPR may not be important for macrophage differentiation. In other words, while hematopoietic precursor HL-60 cells are released from ER stress during neutrophil or macrophage differentiation, cell-lineage specific UPR activation is required for neutrophil differentiation.

Effects of inhibition of mitochondrial ATP production on myelocytic differentiation

Because both ER stress sensing and URP activation require ATP [1, 2, 13], we next analyzed whether ATP synthesis inhibition has effects on myelocytic differentiation using oligomycin, an inhibitor of mitochondrial ATP supply (Figs. 5 and 6).

Oligomycin significantly reduced mitochondrial ATP production to one-tenth and half during neutrophil and macrophage differentiation compared with ATRA- or PMA-treated cells, respectively, whereas no significant difference was observed between ATRA or PMA



	Day 1	Day 2	Day 3	Day 4 (%)
ATRA (A)	100	100	100	100
A + DMSO	97	73	79	118
A + O 0.3 μM	51	38	40	63
A + O 1 μM	43	30	32	51
A + O 3 μM	33	16	16	15

	Day 1 (%)
PMA (P)	100
P + DMSO	106
P + O 0.3 μM	62
P + O 1 μM	63
P + O 3 μM	55

Fig. 5. Inhibitory effect of ATP production by oligomycin during neutrophil and macrophage differentiation. (a) ATP production during neutrophil differentiation with and without oligomycin. (b) ATP production during macrophage differentiation with and without oligomycin. The relative value is normalized with protein amounts. The tables in (a) and (b) show the percentages of ATP production compared with either ATRA- or PMA-treated cells, which was taken as 100 %. NT, non-treated; A, ATRA; P, PMA; O, oligomycin. *, † P<0.05, **, †† P<0.01, ***, ††† P<0.001. Asterisk(s), v.s. NT; dagger(s), v.s. ATRA.

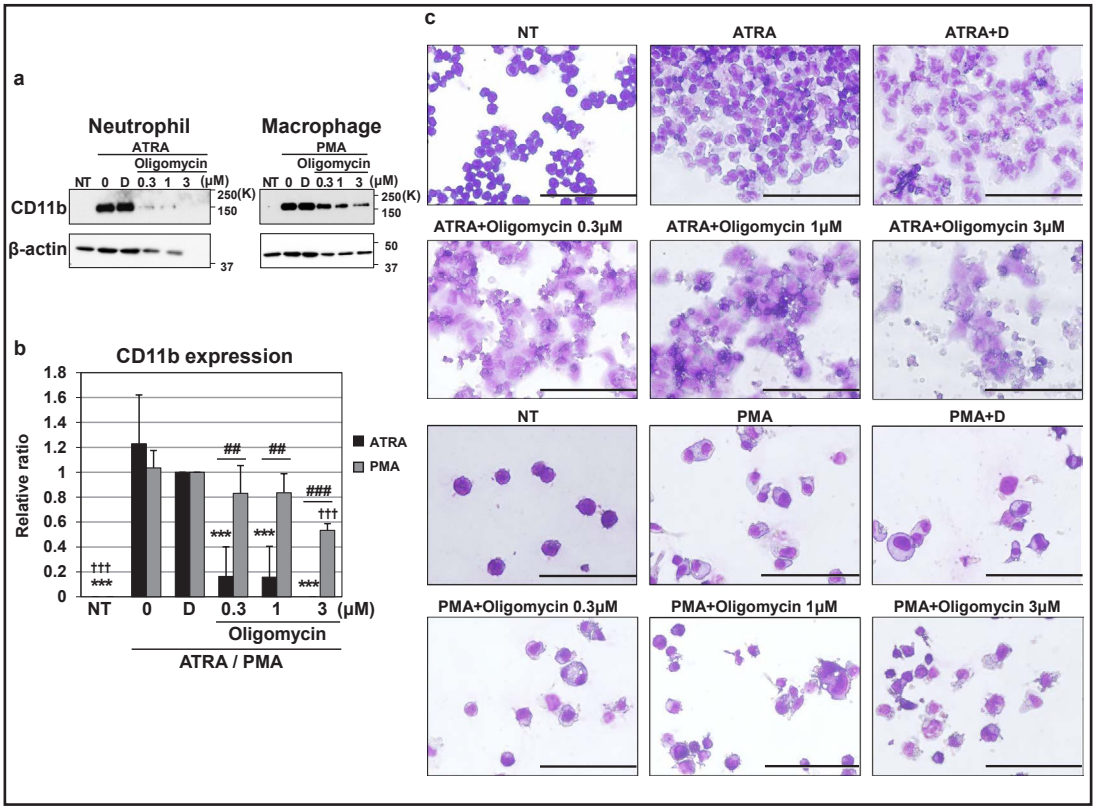


Fig. 6. Effect of ATP depletion by oligomycin on neutrophil and macrophage differentiation (a) CD11b expression during neutrophil and macrophage differentiation with and without oligomycin. CD11b expression was analyzed at day 4 for ATRA treatment and at day 1 for PMA. (b) Relative ratio of CD11b expression in (a). The relative ratio is normalized using "D" as 1. O, Oligomycin; NT, non-treated; A, ATRA; D, inducer + DMSO. * P<0.05; **, ## P<0.01; ***, ###, †††, P<0.001. Asterisk(s), v.s. ATRA + D; dagger(s), v.s. PMA + D. (c) Morphological confirmation of oligomycin effects on neutrophil and macrophage differentiation by Wright-Giemsa staining. Scale bar, 100 μm.

treatment with and without DMSO (Fig. 5). CD11b expression also markedly decreased during neutrophil and macrophage differentiation (Fig. 6). Interestingly, the reduction in CD11b expression was higher during neutrophil differentiation than during macrophage differentiation (Fig. 6b). Additionally, in morphological analysis as shown in Fig. 6c, oligomycin inhibited neutrophil differentiation at the stage of promyelocyte and resulted in inducing cell death. However, apoptosis rate was dose-dependently reduced by oligomycin treatment and cleaved Caspase-3 was not detected (Fig. 4b, d, e).

By contrast, many cells treated with PMA plus oligomycin could differentiate into macrophage (Fig. 6b). But apparent apoptosis was not detected in PMA treated cells by Wright-Giemsa staining and cleaved caspase 3 was also not detected by western blot analysis. These findings indicate that reduction in mitochondrial ATP production significantly influences neutrophil differentiation, but not macrophage differentiation, suggesting that mitochondrial ATP synthesis is essential for neutrophil differentiation.

Based on these findings, we further examined the role of mitochondrial ATP in UPR during myelocytic differentiation. We tested whether UPR is inhibited by oligomycin treatment. In neutrophil differentiation, ATF6 and PERK-ATF4 were not affected by oligomycin treatment at day 1 (Fig. 7). However, XBP-1 activation was abolished by oligomycin at day 4 of ATRA treatment. Moreover, BiP expression was increased in oligomycin-treated HL-60 cells at the same level as that in non-treated HL-60 cells. Thus, these results indicate that ATP depletion by oligomycin inhibited neutrophil differentiation via the impairment of IRE1α-XBP1

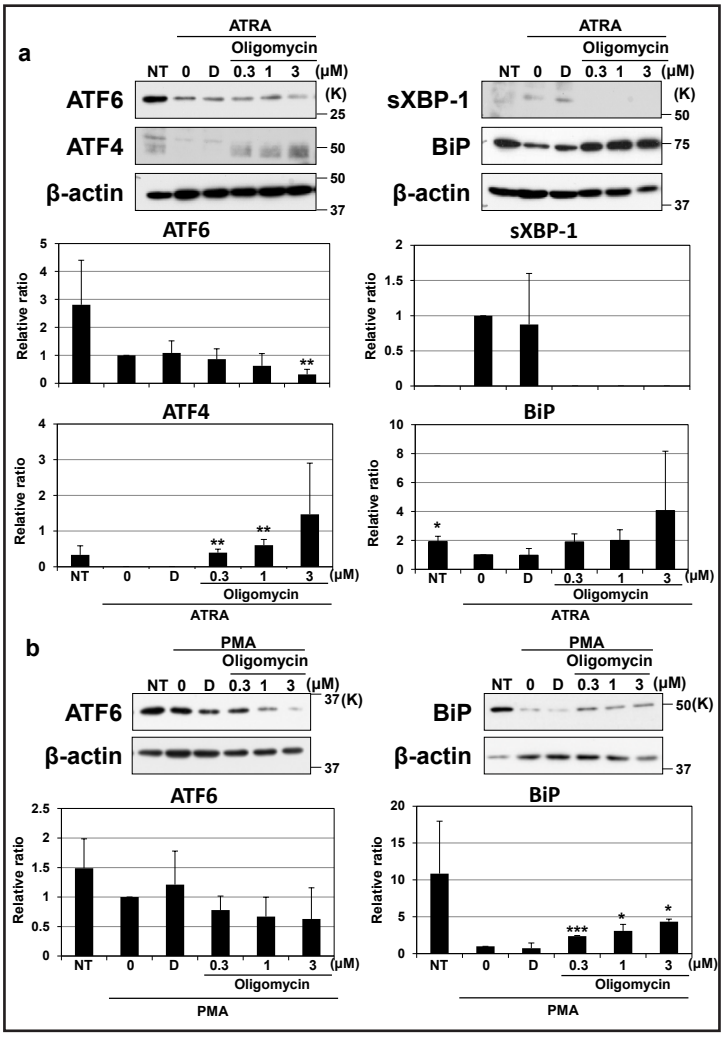


Fig. 7. Effect of ATP depletion by oligomycin on UPR expression during neutrophil and macrophage differentiation. (a, b) Inhibition of UPR pathways (ATF6, PERK, and IRE1α) during neutrophil and macrophage differentiation by oligomycin. Cleaved ATF6 represents marker for ATF6 activation, ATF4 for PERK activation, and spliced XBP-1 (sXBP-1) for IRE1α-XBP1 pathway activation at day 4 for ATRA treatment (a) and at day 1 for PMA (b) by western blot analysis. β-actin is shown as a loading control. Data of western blot analysis are represented using graphs. Except for ATF4 in neutrophil differentiation, the relative ratio is estimated using the value of ATRA-treated or PMA-treated HL-60 cells as 1. Data of ATF4 in neutrophil differentiation are shown as values divided by β-actin because the value of ATF4 in ATRA-treated HL-60 was zero, which could not be used to estimate data. NT, non-treated; A, ATRA; P, PMA; D, DMSO (vehicle of oligomycin) with ATRA or PMA; O, oligomycin. N = 3. * P<0.05, ** P<0.01, *** P<0.001.

activation and induced high ER stress at the similar level to that in non-treated HL-60 cells. In macrophage differentiation, ATF6 expression was not significantly affected by oligomycin treatment. BiP expression was increased by oligomycin, but it was not as high as that in non-treated HL-60 cells.

Discussion

In this study, we examined the functional link between ER stress/UPR and hematopoietic differentiation *in vitro* using bipotent HL-60 cells. Specifically, we focused on the relationship between ATP supply from mitochondria and ER stress/UPR. Our findings on ER stress/UPR dynamics during neutrophil and macrophage differentiation are summarized in Fig. 8.

ER stress as a regulator in neutrophil and macrophage differentiation

Under a standard culture condition, HL-60 cells highly expressed BiP, a marker for ER stress. As shown in Fig. 1, BiP expression was reduced to a minimum level during neutrophil differentiation, whereas it nearly completely disappeared during macrophage differentiation. These findings suggested that ER stress might inhibit myeloid differentiation. Consistent with these findings, 4-PBA, a chemical chaperone [12], was found to induce CD11b expression and morphological differentiation with reciprocal decrease in BiP expression, demonstrating that reduction of ER stress is required for HL-60 cell differentiation (Fig. 1c, d). Intriguingly,

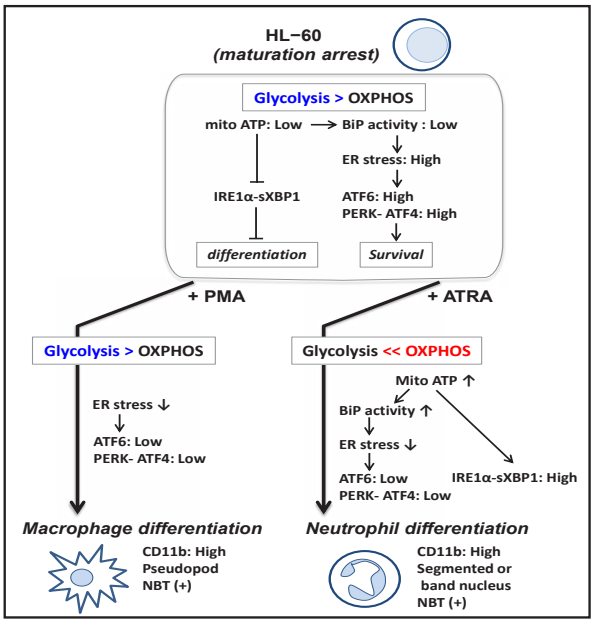


Fig. 8. Hypothetical model of a functional link between bioenergetics and ER stress/UPR in undifferentiated and myeloid-differentiating states of HL-60 cells (Upper part) In non-treated HL-60 cells, ATP production relied on glycolysis. The low ATP supply from mitochondria (mito ATP) promotes high ER stress, as evidenced by strong BiP expression, causing impairments in BiP activation and protein folding. High ER stress level impairs myeloid differentiation. However, ATF6 and PERK, which can function under the condition of low ATP amount, may support HL-60 cell survival against high ER stress. (The lower right part) During neutrophil differentiation in response to ATRA, mitochondrial ATP supply by OXPHOS increases through unknown mechanism. Subsequently, higher ATP supply from mitochondria triggers the following two responses: 1) Reduction in ER stress, which possibly occurs by increased BiP activation due to increased ATP supply; reduced ER stress then reduces ATF6 and PERK-ATF4 activity. 2) XBP-1 activation via IRE1α, which requires more ATP for its own activation. Both PERK-ATF4 and ATF6 activation levels decrease at the early stage, whereas XBP1-spliced form level increases at the late stage of neutrophil differentiation. Thus, during differentiation, HL-60 cells released from ER stress acquire the ability to differentiate step-by-step, and activated XBP-1 specifically leads to the neutrophil differentiation of HL-60 cells. (The lower left part) On the other hand, during macrophage differentiation after PMA treatment PKC signal may induce the reduction in ER stress by unknown mechanism(s). In macrophage differentiation, ATP may be supplied mainly from glycolysis. Mitochondrial ATP production may be similar to that in undifferentiated HL-60 cells. Both reduction in ER stress and induction of key factor(s) by PKC signal may trigger macrophage differentiation in HL-60 cells.

morphological analyses shown in Fig.1, 3 and 6 revealed that 4-PBA-treatment induced partial differentiation of neutrophil such as myelocyte, metamyelocyte, and a few of segmented neutrophil, compared to the cells treated with ATRA only, showing more matured neutrophil with segmented and band nucleus. From these results, maturation arrest of HL-60 might be caused by not only impairment of ER stress-UPR axis but also unknown disturbance of metabolic regulation that is restored by retinoic acid. Taken together, based on our findings, we thought that high ER stress levels in undifferentiated HL-60 cells could function as a negative regulator for myeloid differentiations (Fig. 8).

Lineage-specific UPR activation in myelocytic differentiation

During neutrophil differentiation of HL-60 cells, both cleaved ATF6 and ATF4 expressions were reduced to minimum levels at the early differentiation phase and activation of XBP1 expression occurred at the later phase (Fig. 2). On the other hand, in macrophage differentiation, cleaved ATF6 expression was reduced to minimum levels, whereas ATF4 expression was lost and XBP-1 was not activated. About the reduction of uncleaved ATF6, it has been reported that ER stress regulates expression of *ATF6* mRNA through cleaved ATF6 level [14]. Therefore, it was thought that decreased ER stress reduces cleaved ATF6 level, and then suppress full length ATF6 protein expression through regulating its mRNA expression. Importantly, our findings suggested that lineage-specific UPR activity is required during myeloid differentiation, which is consistent with the findings in previous studies on hematopoietic differentiation [5, 15, 16].

For neutrophil differentiation, all UPR inhibitors used in this study inhibited neutrophil differentiation (Fig. 3), and blocking of mitochondrial ATP synthesis by oligomycin impaired

neutrophil differentiation via the inhibition of XBP-1 activation (Figs. 6 and 7). These results suggest that UPR activations, especially that of XBP-1, through mitochondrial ATP supply is critical for neutrophil differentiation.

In addition, we found the unique switching profile of UPR from reducing ATF6 and PERK expression at the early phase of neutrophil differentiation to increasing XBP-1 expression at the late phase (Fig. 2). Consistent with these findings, a previous study demonstrated that ATF6 increases unspliced *Xbp-1* mRNA and that PERK-ATF4 cascade increases IRE1α expression; thus, both ATF6 and PERK may promote XBP-1 activation [17]. Therefore, together with our experimental data, we hypothesize that the integration mechanism of two UPR pathways of ATF6 and PERK lead to the enhancement of XBP-1 activation, which is critical for neutrophil differentiation (Fig. 8). However, this scenario remains to be determined.

Previous studies demonstrated that XBP-1 expression was related to hematopoietic differentiation [5, 18]. Bettigole et al. demonstrated that eosinophil differentiation failed in *Xbp-1*-KO mice, while neutrophil differentiation was not affected [5]. However, Shen et al. argued that the functional differentiation of neutrophil in *Xbp-1*-KO mice was not determined [18]. In addition, Bettigole et al. checked $CD11b^+Ly6G^+$ cells as differentiated neutrophils in *Xbp-1*-KO mice [5]. However, both differentiation markers (CD11b and Ly6G) are expressed from the early stage (promyelocyte) to the late stage (matured neutrophil) in neutrophil differentiation [19]. Consequently, it was suggested that $CD11b^+Ly6G^+$ cells do not fully differentiate into mature neutrophils. In our previous study, we demonstrated that HL-60 cells treated with ATRA *in vitro* for 4 days functionally differentiated and showed phagocytic activity and NBT-reducing capacity [11]. Therefore, we believe that XBP-1 may be essential at the late stage of neutrophil differentiation to become functionally matured neutrophils.

In contrast to neutrophil differentiation, macrophage differentiation was not inhibited by any UPR inhibitors (Figs. 3b and 3c). However, ER stress was significantly reduced during macrophage differentiation (Fig. 1). These findings suggested that ER stress regulates macrophage differentiation similar to neutrophil differentiation and that ER stress is controlled by other systems but not UPR. Further studies are required to prove the hypothesis and identify key endogenous player(s) of macrophage differentiation in ER stress reduction. These players may be downstream targets of PKC and independent of UPR.

Importance of ATP supply from glycolysis and mitochondrial oxidative phosphorylation (OXPHOS)

During neutrophil differentiation, ATP production was decreased gradually in ATRA- and (ATRA+DMSO)-treated cells compared to non-treated cells. This finding was consistent with the previous reports, in which ATP production in neutrophil-differentiated HL-60 cells was shown to be lower than in undifferentiated HL-60 cells [20].

Another interesting finding is the functional link between ATP source and cellular differentiation through ER stress and UPR regulation. Protein folding steps supported by chaperone activity require ATP [21, 22]. Furthermore, BiP, a chaperone and an ER stress sensor, also needs ATP for its activation [1, 23]. A previous study demonstrated that the inhibition of ATP utilization with 2-deoxy-D-glucose increased ER stress [24]. For UPR, IRE1α has an ATP-binding pocket, which requires ATP for its activation [1, 2]. PERK also requires ATP for its activation [25]. On the other hand, ATF6 does not have any ATP-binding sites [2], and no relationship between ATF6 and ATP has been reported.

The cells obtain ATP through glycolysis and/or OXPHOS. Haga et al. demonstrated a possible link between mitochondrial ATP production and UPR activation under glucose deprivation [26]. As shown in Figs. 5 and 6, we demonstrated that neutrophil and macrophage differentiation relies on ATP production from mitochondrial OXPHOS in varying degrees. We found that intracellular ATP contents were significantly decreased during neutrophil differentiation in the presence of oligomycin in a dose-dependent manner. Application with 3 μM of oligomycin reduced ATP content by approximately 90% after ATRA treatment (Fig. 5a). On the other hand, ATP content decreased by about 40% after adding 0.3 μM of oligomycin, although higher doses did not further reduce ATP content during macrophage

differentiation (Fig. 5b). High mitochondrial dependency was observed during neutrophil, but not macrophage differentiation. Consistent with these observations, a previous report demonstrated that HL-60ρ0 cells, in which mitochondria are depleted and only glycolysis is the ATP source, could differentiate into macrophages but not neutrophils [27]. These findings indicated that ATP for ER stress/UPR, especially XBP1 activation, is mainly supplied from mitochondria for neutrophil differentiation, whereas glycolysis-derived ATP is sufficient for macrophage differentiation (Fig. 8). For neutrophil differentiation, BiP chaperone could be activated by mitochondrial ATP supply, and protein folding may proceed smoothly (Fig. 8, right lower part). IRE1α activation could also occur by ATP supply to support neutrophil differentiation. However, it is essential to clarify how ATRA increases mitochondrial ATP production (mito ATP). In contrast, ATP supplied from glycolysis was sufficient to induce macrophage differentiation of HL-60 cells (Fig. 8, left lower part). One possible explanation for different ATP demand between macrophage and neutrophil differentiation is that IRE1α activation requires relatively higher ATP amounts than PERK activation. PERK may be activated with low level of ATP supplied from glycolysis. On the other hand, IRE1α may need high ATP amounts which derived from mitochondrial OXPHOS. It is essential to determine the ATP sensitivity of each UPR pathway to develop small chemical molecules targeting each UPR activity.

UPR-requirement for cell survival during neutrophil differentiation

As shown in Fig. 3d and Fig. 4, apoptotic cells were detected with ATRA or ATRA plus UPR inhibitor treatment. However, they were not detected in ATRA + 3μM oligomycin or PMA-treated groups. The apoptotic percentage in ATRA-treated cells increased to approximately 7.5 %. Retinoic acid has been reported to induce extrinsic apoptosis mediated by TRAIL induction and subsequent caspase-3 activation [28, 29]. In addition, caspase-independent apoptosis mediated by calpain was also reported in human neutrophil [30]. As shown in Fig. 4c to e, cleaved caspase 3 was hardly detected in ATRA-treated cells as the same as non-treated cells, suggesting that ATRA-induced apoptosis is mainly caused by caspase-3 independent. These data suggested that ATRA-induced apoptosis in HL-60 cells might be through calpain as a possible mechanism.

Intriguingly, we observed increased apoptosis specifically by AEBSF and 4μ8C, but not GSK, in the analysis of HL-60 cells treated with ATRA plus UPR inhibitors (Fig. 4a). These findings indicated that blocking the UPR signals may increase apoptosis of HL-60 cells through enhancing ER-stress. In other words, UPR signals are critical for cell survival.

UPR has two modes. One is survival pathway through enhancement of chaperones or ERAD in response to mild ER stress, and other is apoptotic pathway through CHOP/caspase-3 and ATG proteins in response to severe/chronic ER stress [31-35]. Fig. 1 showed that the BiP level was reduced during neutrophil differentiation, indicating that ER stress was mitigated, and Fig. 2 indicated the switching of the UPR pathway in neutrophil differentiation from PERK and ATF6 in the early phase to IRE1α-XBP1 in the late phase. These findings suggested that ATRA-induced neutrophil differentiation of HL-60 cells requires activation of all UPR pathways to reduce ER stress and to survive. Therefore, treatment with UPR inhibitors was meant to increase ER stress, thereby it was thought that apoptosis was enhanced. In summary, UPR has a critical role in survival of HL-60 cells by reducing ER stress during neutrophil differentiation. However, we need study to further dissect the roles of phase-dependent multifaceted UPR signals for cell survival during ATRA-induced neutrophil differentiation.

Additional interesting phenomena are as follows; PMA did not induce apoptosis, and oligomycin inhibited ATRA-induced apoptosis. First, in a previous study, PMA treatment showed the reduced apoptotic rate compared to HL-60 cells treated with thapsigargin, an ER stress inducer, or 4-bromo-calcium ionophore at the similar level to untreated HL-60 cells [36]. It was demonstrated that PMA treatment could induce and activate Bcl2 family members including Mcl1 that are associated to anti-apoptotic activity through PKC activation [37-39]. However, the detail mechanisms remain unclear, so we need to further clarify. Second, oligomycin reduced ATRA-induced apoptosis. It has been also reported that

regulation of cell death types, either apoptosis or necrosis, is depending on intracellular ATP level. We observed that oligomycin treatment significantly reduced cellular ATP amounts by inhibiting mitochondrial ATP production and it concomitantly reduced apoptotic rate as shown in Fig. 4 and Fig. 5a. This finding clearly demonstrates the importance of mitochondria linking energy metabolism and ER stress/UPR in ATRA-induced neutrophil differentiation.

Relationship between ER stress/UPR and hematopoietic diseases with differentiation arrest

Previous reports on congenital neutropenia have suggested a link between ER stress/UPR, mitochondrial ATP production, and neutrophil differentiation [40-44]. UPR dysfunction and increased ER stress occur in cells with a mutation of the *elastase, neutrophil expressed* (*ELANE*) gene, which encodes an abnormal neutrophil elastase (NE), impairing neutrophil differentiation [40, 41]. Additionally, mutation of the ubiquitous glucose-6-phosphatase subunit α gene (*G6PC3*) also leads to neutrophil defects. This mutation affects protein glycosylation by increasing ER stress by compromising glycosylation without increasing serum glucose concentration [42, 43]. These reports suggested that both maintaining UPR and controlling ER stress are important for neutrophil differentiation in a stage- and intensity-specific manner. Regarding the stage-specific activation of UPR, spliced XBP-1 was induced during B-cell differentiation using LPS stimulation [44]. Furthermore, in reticular dysgenesis (RD) (OMIM #267500, http://www.omim.org/entry/267500), the myeloid precursor cells with AK2 deficiency cannot produce ATP in mitochondria, resulting in defective ATP transport from the mitochondria to the ER during neutrophil differentiation. Therefore, in AK2-deficient myeloid precursor cells, IRE1α-sXBP1 activation might be impaired through an insufficient mitochondrial ATP supply. As a consequence, abnormal ER stress and UPR dysfunction could block neutrophil differentiation in RD patients, such as that shown in patients with neutropenia due to *ELANE* mutation [40, 41]. In contrast, macrophage differentiation can be maintained by ATF6 activation even in the absence of mitochondrial ATP supply because ATF6 activation does not require ATP.

Conclusion

In this study we demonstrated that stage-specific regulation of ATF6, PERK, and IRE1α activation occurs during neutrophil differentiation of HL-60 cells and that mitochondrial ATP supply may be essential for IRE1α activation. Therefore, myeloid precursor cells may reduce ER stress during both macrophage and neutrophil differentiation, and XBP-1 activation may promote neutrophil differentiation. Only ATF6, which does not require ATP, was activated for macrophage differentiation. Because sufficient ATP can be supplied from glycolysis, energy homeostasis can be maintained without OXPHOS during macrophage differentiation. In conclusion, we demonstrated a close correlation between mitochondrial ATP production and neutrophil differentiation through stage-specific activation of UPR branch, such as XBP-1, and reduction in ER stress. However, our study did not demonstrate how ATRA and PMA control the switching of cell fate through ATP supply from glycolysis or mitochondria. Further, the detailed molecular mechanisms of myeloid differentiation through reduced ER stress and UPR activation is still unclear. Further studies are required to solve these pertinent questions, which will provide the target to develop new therapies for mitochondrial diseases, such as reticular dysgenesis.

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Disclosure Statement

The authors declare to have no competing interests.

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