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Metformin induces differentiation in acute promyelocytic leukemia by activating the MEK/ERK signaling pathway

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ABSTRACT

Recent studies have shown that metformin, a widely used antidiabetic agent, may reduce the risk of cancer development. In this study, we investigated the antitumoral effect of metformin on both acute myeloid leukemia (AML) and acute promyelocytic leukemia (APL) cells. Metformin induced apoptosis with partial differentiation in an APL cell line, NB4, but only displayed a proapoptotic effect on several non-M3 AML cell lines. Further analysis revealed that a strong synergistic effect existed between metformin and all-trans retinoic acid (ATRA) during APL cell maturation and that metformin induced the hyperphosphorylation of extracellular signal-regulated kinase (ERK) in APL cells. U0126, a specific MEK/ERK activation inhibitor, abrogated metformin-induced differentiation. Finally, we found that metformin induced the degradation of the oncoproteins PML-RARα and c-Myc and activated caspase-3. In conclusion, these results suggest that metformin treatment may contribute to the enhancement of ATRA-induced differentiation in APL, which may deepen the understanding of APL maturation and thus provide insight for new therapy strategies.

1. Introduction

Metformin (1,1-dimethylbiguanide hydrochloride) is an oral hypoglycemia agent commonly used for the treatment of type 2 diabetes mellitus [1]. Metformin displays various advantageous metabolic effects. It decreases blood glucose by inhibiting gluconeogenesis in hepatic cells and stimulating glucose uptake in muscle cells. It also reduces the circulating insulin levels as an "insulin sensitizer" by enhancing signaling through the insulin receptor [2,3]. In addition to its antidiabetic properties, metformin has recently attracted attention due to its anticancer effects. Some studies have shown that metformin induces the growth inhibition of breast cancer cells by activating AMPK, which is a direct substrate of liver kinase B1 (LKB1), a classic tumor suppressor [4]. Additionally, population studies have suggested that treatment with metformin is associated with a lower risk of cancer development in diabetic patients compared with those taking other diabetes drugs such as sulfonylureas and insulin [5,6]. The combined action of metformin and classic chemotherapeutic agents has been shown to be effective in both cancer prevention and treatment. Metformin treatment combined with paclitaxel, cisplatin or doxorubicin considerably reduces tumor growth compared with single agent treatment in mice [7,8]. Taken together, these observations from cell culture and pre-clinical studies indicate that metformin may function as an adjunct to other conventional cancer therapies.

Acute promyelocytic leukemia (APL) is a deadly disease characterized by a specific translocation t(15;17), which generates the promyelocytic leukemia/retinoic acid receptor α (PML/RARα) fusion protein and induces differentiation blockade at the promyelocytic stage [9,10]. Fortunately, treatment with all-trans retinoic acid (ATRA), a differentiation inducer, has demonstrated high rates of clinical complete remission (CR) in APL patients when combined with chemotherapy [11]. However, a significant percentage (30–40%) of patients relapse with resistance to further ATRA treatment [12]. A recent discovery indicates that arsenic trioxide (As_2O_3) induces durable remission in APL patients with relapse after ATRA and chemotherapy treatment [13,14]. ATRA induces the terminal differentiation of APL cells both in vitro and in vivo via the activation of nuclear retinoic acid and retinoid-X receptors, which is accompanied by the degradation and modulation of the PML-RARα fusion protein [15,16]. The changes in cell signaling, involving pathways such as the MEK/ERK pathway, are required to promote retinoid-induced myeloid differentiation [17]. As_2O_3 also induces the rapid modulation and degradation of PML-RARα proteins

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[13]. Therefore, APL represents a successful model of fusion protein-targeted drug therapy.

Although little population study data exist showing the association between metformin treatment and the risk of APL in diabetic patients, two recent reports have emphasized that metformin exerts cytotoxic effects on both AML and T-ALL cells via the LKB1/AMPK tumor-suppressor pathway [18,19]. In the present study, we confirmed the proapoptotic effect of metformin on both AML and APL cells. Interestingly, metformin-induced differentiation was observed selectively in APL cells; therefore, the NB4 cell line was selected as an in vitro model to investigate the possible cellular and molecular mechanisms of metformin as a differentiation inducer in the treatment of APL. The MEK/ERK signaling pathway was demonstrated to play a role in metformin-induced differentiation. Moreover, we demonstrated that the interaction between metformin and ATRA in the induction of differentiation in NB4 and primary APL cells was synergistic. These results demonstrate a new mechanism that may contribute to the antileukemia effect of metformin.

2. Materials and methods

2.1. Patients

Bone marrow samples were obtained from eight newly diagnosed APL patients with t(15;17) who were enrolled at the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences. All patients provided informed consent. The diagnosis and leukemia classification were based on 2008 World Health Organization criteria.

2.2. Cell culture and reagents

AML cell lines (Kasumi-1, SKNO-1, HL-60 and KG-1a) and the APL cell line (NB4) were routinely maintained in RPMI-1640 medium with 10% fetal bovine serum. SKNO-1 cells were supplemented with 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). Bone marrow mononuclear cells were cultured in Iscove's modified Dulbecco's media supplemented with 15% fetal bovine serum.

Metformin and ATRA were purchased from Sigma–Aldrich (St. Louis, MO, USA). U0126 was obtained from Cell Signaling Technology (Boston, MA, USA).

2.3. Apoptosis assessment by annexin V staining

Cells were collected and analyzed for apoptosis with an Annexin V-FITC kit (BD Biosciences) according to the manufacturer's instructions while simultaneously assessing membrane integrity with propidium iodide (PI) exclusion (BD Biosciences).

2.4. Differentiation assay by immunophenotype, morphology and nitroblue tetrazolium (NBT) reduction

For cell surface marker analysis, the PE-conjugated mouse antibody for human CD11b (Becton Dickinson) was used for the FACScalibur flow cytometric analysis. A PE-conjugated mouse isotype-matched IgG antibody (Becton Dickinson) was used as a negative control to determine the background fluorescence.

To assess cell morphology changes, the cells were prepared on a glass slide with a cytospin apparatus and stained with Wright solution. The images were captured using a Nikon Eclipse 50i microscope (Nikon Inc., Melville, NY, USA).

Nitroblue tetrazolium (NBT) reduction was performed as previously reported [20]. The percentage of NBT-positive cells with formazan deposits in the cytoplasm was determined by microscopically quantifying at least 300 cells per experimental condition.

2.5. Subcellular localization analysis using immunofluorescence

Immunofluorescent staining of the N-terminal region of PML was performed according to the PML antibody manufacturer's instructions (Abcam). Fluorescence was observed using a Leica confocal laser microscope.

2.6. Western blot analysis

Protein lysate preparation and Western blotting were performed according to the manufacturers' instructions. Anti-phospho-p44/42 ERK (Thr202/Tyr204) rabbit monoclonal antibody (mAb), anti-p44/42 ERK rabbit mAb, anti-Bcl-2 rabbit mAb, anti-c-Myc rabbit mAb, anti-Bcl-xL rabbit mAb, anti-caspase-3 antibody and anti-cleaved caspase-3 (Asp175) rabbit mAb were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-PML and anti-RARα polyclonal antibodies were purchased from Abcam (Cambridge, MA, USA). An anti-actin mAb (Sigma) was used as an internal control. The immunoreactive proteins were visualized using the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL, USA) at similar exposure times.

2.7. Statistical analysis

At least three repetitions were performed for each experiment. The significance of the differences between the two groups was determined using Student's t test. All analyses were carried out using the SPSS 13.0 software package (SPSS, Chicago, IL, USA). $p < 0.05$ was deemed statistically significant.

3. Results

3.1. Metformin induces apoptosis in APL and AML cells, whereas it selectively induces differentiation in the APL cell line, NB4

A previous study showed that metformin induces apoptosis in primary AML cells [18]. We confirmed the proapoptotic effect of metformin on various AML and APL cell lines (Fig. 1A). To further assess whether metformin treatment also induced the differentiation of leukemia cells, the expression levels of the cell surface marker CD11b were analyzed. Interestingly, the proportion of $CD11b^+$ NB4 cells increased in a dose- and time-dependent manner (Fig. 1C and D), whereas no changes were found in other non-M3 AML cell lines (Fig. 1B). Morphologically, both apoptotic and partially differentiated cells were visible (Fig. 1E). A certain proportion of cells presented with some differentiated characteristics, such as an excentric nucleus, a reduced nuclei to cytoplasm ratio, the appearance of granules in the cytoplasm and a less basophilic cytoplasm (Fig. 1E); however, these cells resembled myelocytes and metamyelocytes rather than granulocyte differentiation following ATRA treatment (Fig. 1E). In accordance with the morphological analysis, there was no enhancement of NBT reduction after metformin treatment (Fig. 1F), which indicates that metformin did not sufficiently induce the terminal differentiation of NB4 cells.

3.2. Synergism of metformin and ATRA in inducing the differentiation of NB4 and primary APL cells

To further understand the differentiation-inducing effect of metformin, NB4 cells were used as a model. The cells were pretreated with various concentrations of metformin (0.01–1 mM) for 48 h and subsequently cultured in medium in either the

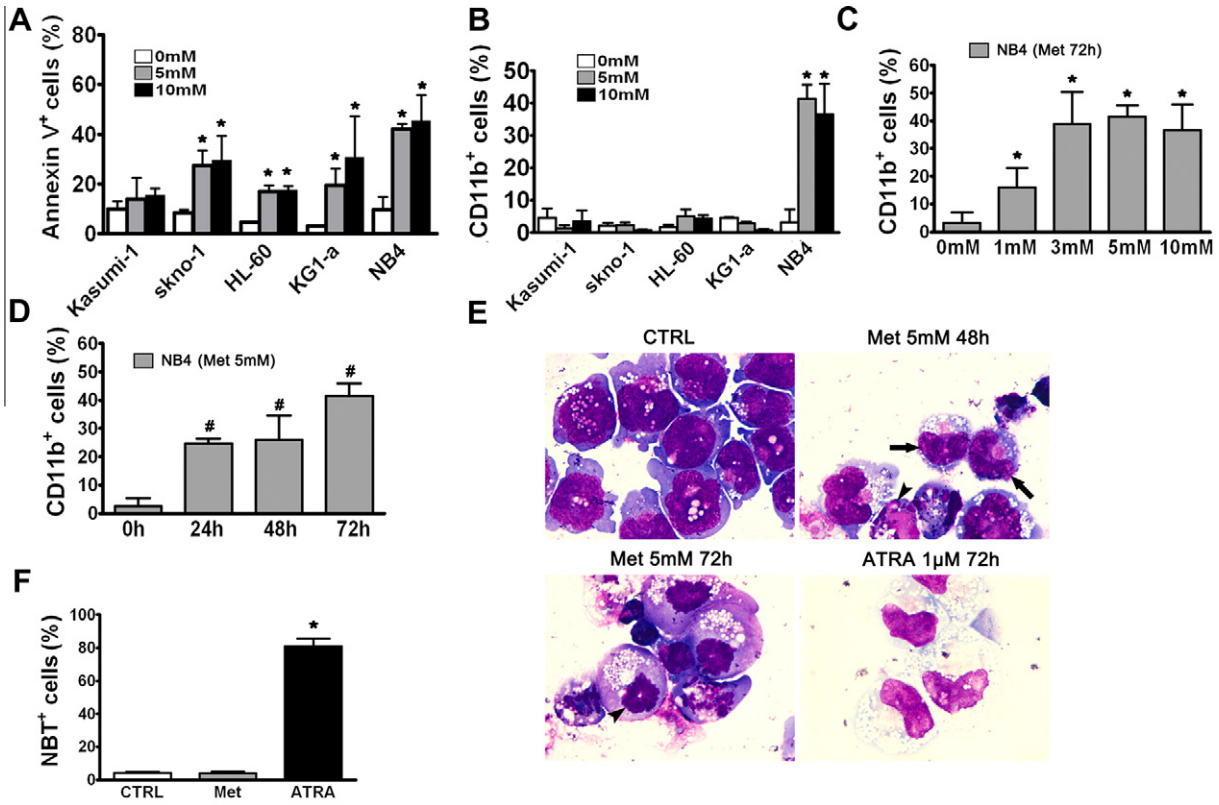

Fig. 1. The effects of metformin on both apoptosis and differentiation in AML and APL cell lines. (A and B) AML cell lines (Kasumi-1, SKNO-1, HL-60 and KG-1a) and an APL cell line (NB4) were treated with the indicated concentrations of metformin for 72 h. Apoptosis was evaluated by FITC-conjugated annexin V binding (A), and differentiation was assessed by measuring CD11b membrane expression levels (B) using flow cytometry. The differentiation of NB4 cells treated with the indicated concentrations of metformin for 72 h (C) or 5 mM metformin for the indicated time periods (D) was assessed as described for panel B. (E) The morphological changes in NB4 cells treated with 5 mM metformin or 1 μM ATRA for the indicated times. Arrows indicate the excentric nucleus, whereas arrowheads highlight condensed apoptotic chromatin. (F) NB4 cells were incubated with 5 mM metformin or 1 μM ATRA for 72 h. Differentiation was evaluated by an NBT assay, as described in Section 2. The results represent the mean ± SD of three independent experiments for each cell line. CTRL: control, Met: metformin. $^{*}p < 0.05$ compared to the untreated groups and $^{\#}p < 0.05$ compared to the metformin-treated group for 0 h.

presence or absence of physiological concentrations of ATRA (1–10 nM) for 72 h. An immunophenotypic analysis showed that a slight increase in CD11b expression levels was observed in the combined group with low concentrations of metformin (0.01–0.1 mM) and ATRA compared with those treated exclusively with ATRA (Fig. 2A). The pretreatment of NB4 cells with a relative high concentration of metformin (1 mM) followed by physiological concentrations of ATRA induced significant cell differentiation with a high proportion of $CD11b^+$ cells compared with those without metformin pretreatment (Fig. 2A); these findings were further supported by the NBT assay results (Fig. 2B).

Due to low proliferation pattern of primary APL cells in vitro, we used an increased concentration of metformin (5 mM) and ATRA (1 μM) to identify the synergistic effect of these drugs on primary APL cells. Similarly, we observed an enhanced effect of differentiation in fresh cells from four de novo APL patients (Fig. 2C).

3.3. The MEK/ERK signaling pathway is essential for the metformin-induced differentiation of NB4 and primary APL cells

Previous studies have indicated that the activation of the MAPK/ERK pathway is involved in the regulation of myeloid cell differentiation [16], which prompted us to investigate whether metformin induces ERK activation during the induction of APL cell differentiation. The cells were cultured in serum-free medium to minimize the influence of serum on ERK activation. Western blot analysis indicated that metformin induced the phosphorylation of MAPK/ERK in a time- and dose-dependent manner (Fig. 3A). To further examine the cause-effect relationship between ERK activation and the induction of differentiation, we performed the following experiments using U0126, a specific MEK/ERK activation inhibitor. We incubated NB4 cells with U0126 for approximately 30 min before adding metformin or ATRA. The results indicated that U0126 effectively inhibited ERK phosphorylation induced by metformin or ATRA, and the metformin- or ATRA-induced granulocytic differentiation was consistently inhibited, as assessed by decreased levels of CD11b membrane expression (Fig. 3B). A similar effect was observed in primary APL cells treated with metformin and U0126 (Fig. 3C).

3.4. Metformin induces the relocalization and degradation of the PML-RARα fusion protein and inhibits the synthesis of oncogenic proteins in NB4 cells

As described above, metformin specifically induced differentiation in NB4 cells. To analyze the exact mode of the selective action of NB4 cells, an immunofluorescence analysis using an anti-PML antibody was performed on HL-60 and NB4 cells. Untreated HL-60 cells, which displayed a normal PML distribution pattern and exhibited 10–20 speckles within the nucleus, were used as control (Fig. 4A), whereas untreated NB4 cells revealed a large amount of micropunctate staining in the nucleus and cytoplasm (Fig. 4A), corresponding to the abnormal staining pattern of PML-RARα and PML-RARα/PML heterodimers [21]. Upon either metformin or

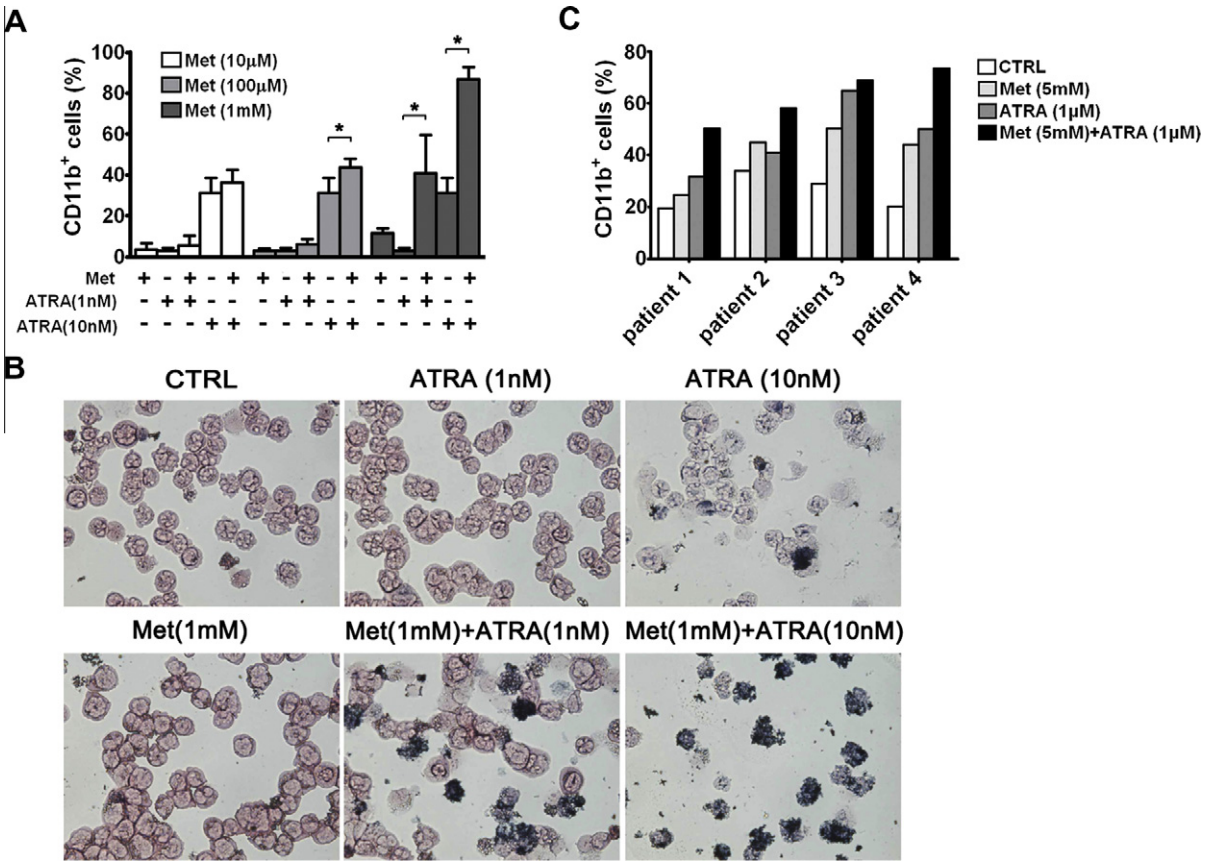

Fig. 2. The synergism of metformin and ATRA in inducing the differentiation of NB4 cells and primary APL cells. Differentiation was assessed by the cytofluorimetric analysis of CD11b membrane expression levels (A) and an NBT assay (B). The results represent the mean ± SD of three independent experiments. (C) Primary leukemia cells were pretreated with either vehicle or 5 mM metformin for 48 h and then exposed to 1 μM ATRA for 72 h. Differentiation was assessed by the cytofluorimetric analysis of CD11b membrane expression. $^{*}p < 0.05$.

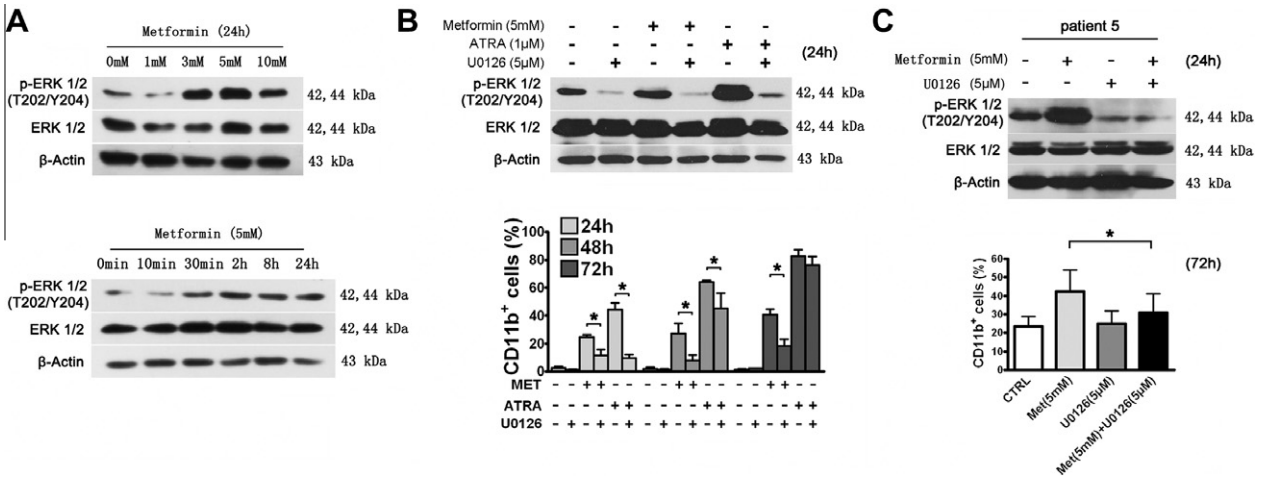

Fig. 3. MAPK/ERK activation is involved in the metformin-induced differentiation of NB4 cells and primary APL cells. (A) NB4 cells cultured in serum-free medium were treated with either metformin at the indicated concentrations for 24 h or 5 mM metformin for different time periods. Cell lysates were assessed by Western blot analysis to examine ERK phosphorylation. (B) NB4 cells were pretreated with either vehicle or U0126 (5 μM) for 30 min and then exposed to metformin (5 mM) or ATRA (1 μM) for the indicated times. Western blotting was performed to assess ERK phosphorylation, and the CD11b membrane expression levels of NB4 cells were assessed by cytofluorimetric analysis. (C) Primary leukemia cells isolated from four de novo APL patients were pretreated with either vehicle or U0126 (5 μM) for 30 min and then exposed to metformin (5 mM) for the indicated times. Patient 5 is representative of four independent experiments. The results represent the mean ± SD of four independent experiments. $^{*}p < 0.05$.

ATRA treatment, normal speckles were restored in NB4 cells (Fig. 4A). An accumulation of PML staining in the perinuclear cytoplasmic region in NB4 cells was observed after metformin or ATRA treatment (Fig. 4A). In accordance with the immunofluorescence analysis, both metformin and ATRA caused the degradation of the PML-RARα fusion protein and the wild-type RARα protein

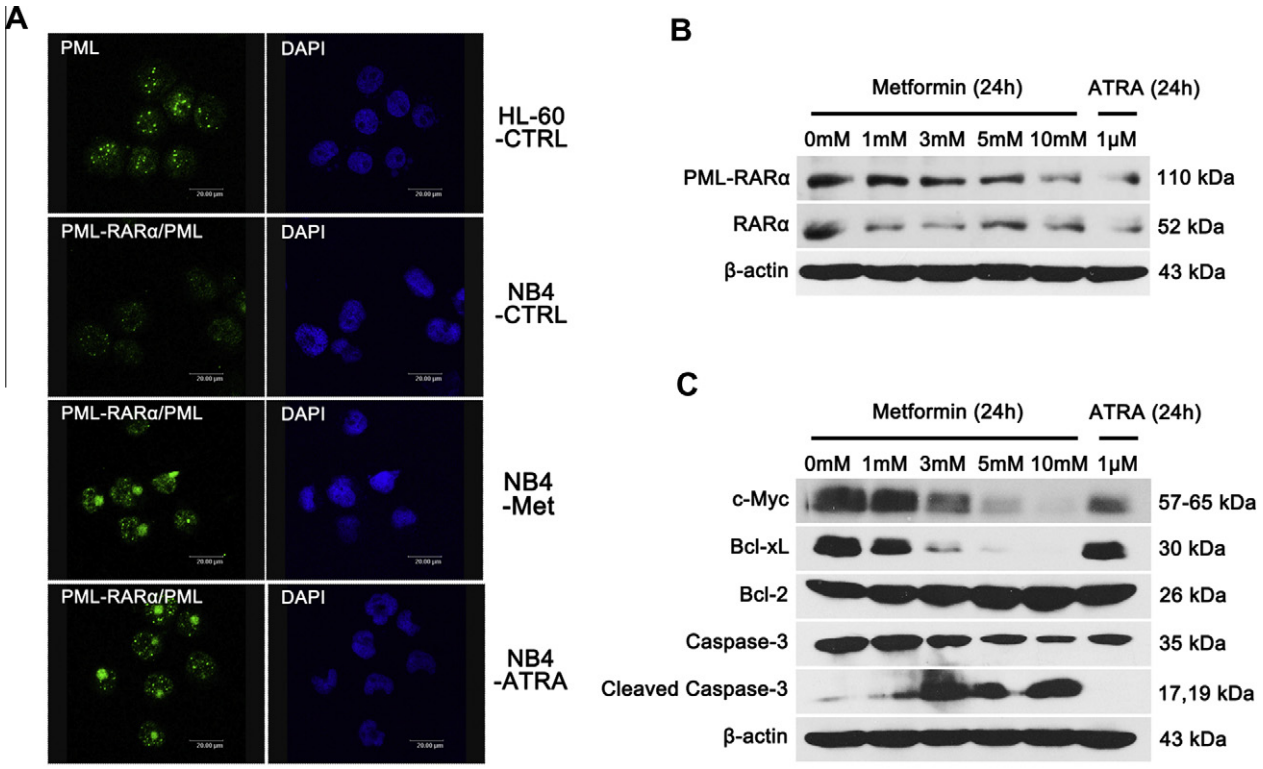

Fig. 4. Metformin induces the relocalization and degradation of the PML-RARα fusion protein and the activation of caspase-3 as well as the reduction of oncogenic protein expression in NB4 cells. (A) NB4 cells were treated with either metformin (5 mM) or ATRA (1 μM) for 24 h. The subcellular localization of PML in HL-60 cells and PML-RARα/PML in NB4 cells was analyzed by immunofluorescence using anti-PML antibodies. (B) Western blot analysis of PML-RARα and RARα expression in NB4 cells treated with the indicated concentrations of metformin or ATRA (1 μM) for 24 h. (C) Western blot analysis of c-Myc, Bcl-xL, Bcl-2, caspase-3 and cleaved caspase-3 expression in NB4 cells treated with the indicated concentrations of metformin or ATRA (1 μM) for 24 h. β-Actin was used as protein loading control.

(Fig. 4B). Further examination revealed that the expression levels of the oncoproteins c-Myc and Bcl-xL were reduced in a dose-dependent manner in metformin-treated NB4 cells, whereas there was no change in the levels of Bcl-2 protein expression (Fig. 4C). Moreover, the emergence of caspase-3-activating cleavage in metformin-treated NB4 cells (Fig. 4C) indicated that metformin-induced apoptosis was associated with the caspase-3 pathway. These findings demonstrate that metformin-induced apoptosis and differentiation in APL cells is involved in both the modulation and degradation of the PML-RARα fusion protein and the reduced expression of oncogenic proteins.

4. Discussion

Recent reports have suggested that metformin displays antileukemic activity in primary AML cells by both reducing AML cell proliferation and inducing apoptosis in primary AML cells [18]. Here, we demonstrated that metformin not only induces apoptosis in APL and AML cell lines but also selectively induces differentiation in an APL cell line and in primary APL cells (Figs. 1 and 2). This observation is of particular importance because it is the first report showing that metformin exerts an anticancer effect by functioning as a differentiation inducer. Both ATRA and As_2O_3 have been established as effective drugs for the treatment of APL. ATRA induces the terminal differentiation of APL cells both in vitro and in vivo, whereas As_2O_3 exerts dose-dependent dual effects on APL cells by inducing both preferential apoptosis at high concentrations (0.5–2 μmol/L) and partial differentiation at low concentrations (0.1–0.5 μmol/L) [22,23]. In the current study, apoptosis and differentiation were simultaneously observed in NB4 cells treated with metformin in a time- and dose-dependent manner (Fig. 1). Moreover, some in vivo studies have demonstrated that the therapeutic effect of As_2O_3 is primarily ascribed to the induction of differentiation [22,24]. During the first 2–3 weeks of a standard As_2O_3 treatment course, differentiated granulocytes at the myelocyte and metamyelocyte stage significantly increase in the bone marrow accompanied by hyperleukocytosis in most of the patients; the leukemia cells then undergo cell death [14]. Therefore, it is believed that the discovery of the differentiation effect of metformin could be more or at least equally as useful as its apoptotic effect for the treatment of APL in vivo.

To further investigate the mechanism underlying the metformin-induced differentiation of APL cells, the effect of metformin in combination with ATRA on both NB4 cells and primary APL cells was examined. We found that 1 mM metformin synergized with ATRA to trigger the maturation of NB4 cells, but 5 mM metformin only displayed an enhanced effect of differentiation in primary APL cells (Fig. 2). The reason is that during in vitro culture, unlike NB4 cells, primary APL cells show little proliferation and the cells are not sensitive to the drug treatment. It has been well proven that metformin treatment combined with classical chemotherapeutic agents is effective in cancer prevention and treatment. Metformin combined with paclitaxel, cisplatin or doxorubicin considerably reduces tumor growth compared with single agent treatment in mice [7,8,25]. Therefore, the differentiation–sensitizing activity of metformin may be one of the most intriguing features of the drug as a potential anticancer agent. This property of metformin closely resembles that of cyclic adenosine monophosphate (cAMP) in NB4 cells. In fact, cAMP treatment renders NB4 cells hypersensitive to ATRA stimulation, even at physiological levels, and the cAMP/PKA pathway is rapidly activated during ATRA-induced APL cell differentiation [26,27]. Interestingly, it has been shown that aden-

ylate cyclase (AC) and protein kinase A (PKA) are involved in the signaling pathway triggered by metformin in HepG2 cells [28]. It is possible that the activity of metformin and ATRA converge at the level of the cAMP/PKA pathway. Further studies on metformin and ATRA-modulated gene expression profiles are therefore required to examine the interaction between the two agents.

Although the precise molecular mechanisms underlying the observed differentiated synergism between metformin and ATRA require further study, the evidence available suggests that the MEK/ERK signaling pathway may be involved. Here, we show that metformin induces the phosphorylation of MAPK/ERK in a time- and dose-dependent manner (Fig. 3A), while demonstrating that the inactivation of MAPK/ERK using U0126 substantially inhibits metformin-induced granulocytic differentiation (Fig. 3B and C). MAPK is essential for the phosphorylation of nuclear RA receptor family members, which appear to influence the ability of retinoids to induce receptor-dependent transcriptional activation, cell growth arrest, and differentiation [16,20,29]. According to our model system, this may be due to the reduction of the differentiation threshold by metformin by increasing the phosphorylation of MAPK/ERK and the corresponding MEK/ERK-dependent nuclear RA receptor phosphorylation status.

In our study, metformin did not affect the expression levels of Bcl-2. However, metformin caused the downregulation of Bcl-xL and c-Myc in NB4 cells (Fig. 4C). The activation of caspase-3, a major regulator of cell apoptosis, may be an intracellular target of the metformin-mediated proapoptotic effect in NB4 cells (Fig. 4C). These findings are consistent with a previous study on AML [18]. Interestingly, we found that metformin restores the subcellular localization of PML and induces the degradation of PML-RARα (Fig. 4A and B), which may be attributed to the repression of oncogenic mRNA translation described above [18]. The PML/RARα fusion protein has been proposed to interfere with nuclear receptor function and myeloid differentiation and has been shown to be involved in the leukemogenesis of APL [30]. In NB4 cells, the degradation of PML/RARα is expected to be responsible for myeloid maturation by relieving the differentiation block [12]. These findings show that PML-RARα may contribute to the selective differentiated effect of metformin on APL cells.

Based on the above date, we suggest the following scenario. Metformin treatment of APL cells activates diverse signaling pathways, including the LKB1/AMPK, MEK/ERK and cAMP/PKA signaling pathways. The activation of MEK/ERK and cAMP/PKA modulates the phosphorylation of nuclear RA receptors, which influences retinoid-induced transcription activation and differentiation. Meanwhile, the LKB1/AMPK signaling pathway inhibits the synthesis of oncogenic proteins such as the PML/RARα oncoprotein. The degradation of PML/RARα liberates DNA binding sites and RARα cofactors, thereby facilitating the ATRA response through the normal receptors. However, due to the deficiency of the RA signal, metformin alone is not sufficient to promote terminal differentiation in APL cells. With the addition of physiological concentrations of ATRA, these two signals cooperate to induce the maturation of APL cells. In summary, our study demonstrates a synergism between metformin and ATRA that triggers the maturation pathway in APL cells, which may be applicable for new differentiation therapy in cancer treatment.

Acknowledgments

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