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Chloroquine enhances cobalt chloride-induced leukemic cell differentiation via the suppression of autophagy at the late phase

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ABSTRACT

We previously reported that moderate hypoxia and hypoxia-mimetic agents including cobalt chloride ($CoCl_2$) induce differentiation of human acute myeloid leukemia (AML) cells through hypoxia-inducible factor-1 α (HIF-1 α), which interacts with and enhances transcriptional activity of CCAAT-enhancer binding factor alpha and Runx1/AML1, two important transcriptional factors for hematopoietic cell differentiation. Here, we show that autophagy inhibitor chloroquine (CQ) increases HIF-1 α accumulation, thus potentiating $CoCl_2$-induced growth arrest and differentiation of leukemic cells. Furthermore, the increased effect of CQ on differentiation induction is dependent of the inhibition of autophagosome maturation and degradation, since this sensitization could be mimicked by the suppression of expression of both lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2). These findings not only provide the evidence that CQ is a sensitizer for $CoCl_2$-induced differentiation of leukemic cells but also possibly propose the new therapeutic strategy for differentiation induction of AML.

1. Introduction

Acute myeloid leukemia (AML), a heterogeneous group of hematological malignancies occurred frequently in adults, is characterized by an accumulation of clonal myeloid progenitor cells that do not differentiate normally [1]. The all-*trans* retinoic acid (ATRA) was successfully applied to the treatment of acute promyelocytic leukemia (APL, a unique subtype of AML) by differentiation induction in the middle of 1980s; and later, arsenic trioxide (ATO) was found to overcome the limitation of ATRA treatment in relapsed or refractory patients [2]. These practices have greatly pushed our understanding on the mechanisms for leukemic cell differentiation [3,4]. Previously, some groups including ours reported that hypoxia and some agents such as cobalt chloride ($CoCl_2$), desferrioxamine (DFO) and Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid induce human AML cells to undergo differentiation through hypoxia-inducible factor-1 α (HIF-1 α) [5–7], a master transcriptional factor of cellular response to hypoxia. Intermittent hypoxia also significantly prolongs the survivals of the transplanted APL mice with differentiation induction and inhibition of infiltration of leukemic cells [8]. More intriguingly, ATRA also rapidly increases HIF-1 α protein, which partially contributes to ATAR-induced leukemic cell differentiation [9]. The ongoing investigations showed that HIF-1 α protein account for AML cell differentiation in its transcriptional activity-independent mechanisms [10]. HIF-1 α and two hematopoietic transcription factors CCAAT/enhancer binding protein alpha (C/EBP α) and Runx1/AML1 interact directly with each other. Such interactions increase the transcriptional activities of C/EBP α and Runx1/AML1 [10–13].

Autophagy plays key roles in the cellular physiological functions such as development, differentiation, antiaging and response to environmental stimuli [14]. This cellular process is also important in the pathogenesis and therapeutic responses of some diseases including cancer [15,16]. Recently, the studies regarding the role of autophagy in the pathogenesis and treatment of leukemia are emerging. We have demonstrated that APL-related fusion protein PML-RAR α enhances constitutive autophagic activity through inhibiting Akt/mTOR pathway and the enhanced activity is critical for the anti-apoptotic function of PML-RAR α [17]. Moreover, several lines of evidence suggest that modulation of autophagic activity and especially suppression of autophagy facilitate the leukemic cell differentiation and/or apoptosis [18,19]. For example, autophagy was reported to play a crucial role in the leukemic cell differentiation induced by ATRA and VitD3 [18,20,21]. Here we

Abbreviations: $CoCl_2$, cobalt chloride; AML, acute myeloid leukemia; HIF-1 α, hypoxia-inducible factor-1 α; CQ, chloroquine; LAMP1 and 2, lysosome-associated membrane proteins 1 and 2; APL, acute promyelocytic leukemia; ATRA, all-*trans* retinoic acid; ATO, arsenic trioxide; DFO, desferrioxamine; shRNA, specific short hairpin RNA; PML-RAR α, promyelocytic leukemia-retinoic acid receptor α; EBSS, Earle's balanced salt solution; hVps34, human vacuolar protein sorting 34; NCF-1, neutrophil cytosolic factor-1; Spp-1, secreted phosphoprotein-1.

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show that chloroquine (CQ), a well-known lysosomotropic agent acts as an inhibitor of autophagy at the late stage [22], increases HIF-1 α accumulation, thus potentiating $CoCl_2$-induced differentiation of leukemic cells, which is dependent of the inhibition of autophagosome maturation and degradation.

2. Materials and methods

2.1. Reagents

$CoCl_2$, CQ and ATRA powders were purchased from Sigma. $CoCl_2$ and CQ were dissolved in ultrapure water to form 100 and 20 mM stock solutions, respectively, and ATRA was dissolved in ethanol as 10 mM stock solution. Rapamycin obtained from Tocris Bioscience was dissolved in DMSO as 1 mM stock solution. EBSS was made according to the media formulations from Invitrogen Company.

2.2. Differentiation assay

Leukemic cell differentiation was evaluated by morphology with Wright's staining, the expression of differentiation antigens CD11b and CD11c and the nitroblue tetrazolium (NBT) reduction test. CD11b and CD11c were measured using fluorescein isothiocyanate (FITC)-labeled anti-CD11b and phycoerythrin (PE)-labeled anti-CD11c, with isotype controls by flow cytometry (BD FACSCliber). The NBT test was performed as previously described [23]. Additional materials and experimental procedures are given in S1 Materials and methods.

3. Results

3.1. $CoCl_2$ alters the autophagic flux during differentiation of NB4 cells

We treated APL cell line NB4 with 50 μM of $CoCl_2$ for different times and EBSS incubation as a positive control (Fig. 1A), and the conversion of LC3-I into LC3-II, a marker of autophagy [24], was examined by western blot. As depicted in Fig. 1A, the substitution of growth medium with the nutrient-free EBSS, an autophagy inducer [25], increased LC3-II in NB4 cells. $CoCl_2$ treatment rapidly increased the endogenous LC3-II protein. A time course analysis revealed that LC3-II protein began to increase by 6 h, reached peak levels at 12 h, and then decreased after 72 h to the basal level. Furthermore, by monitoring the distribution of the fluorescent protein tagged LC3 fusion protein, one could visually track autophagic response by fluorescence microscopy when the cytoplasmically and diffusely distributed LC3-I is converted into the punctate LC3-II [24]. Thus, U_2OS cells were transiently transfected with GFP-LC3 plasmid and subsequently treated with or without 50 μM $CoCl_2$ for 6 h, together with the treatment of rapamycin (another widely used autophagy inducer [24]) at 0.5 μM for 6 h as a positive control. Compared with the control cells, the majority (>70%) of GFP-$LC3^+$ cells treated with $CoCl_2$ demonstrated dramatic transition from the diffuse cytoplasmic pattern to the punctate membrane pattern and this effect was similar to the cells incubated with rapamycin (Fig. 1B). $CoCl_2$ also increased the expression of Beclin-1, another indicator for autophagy initiation that participates in autophagosome formation by interacting with hVps34 [26], which began to appear at Day 3 and appear significant at Day 4 and lasted for up to 6 days. However, $CoCl_2$ treatment failed to decrease the expression level of p62 protein (Fig. 1A), which is reported to selectively incorporate into the autophagosome through direct binding to LC3 and to be efficiently degraded by autophagy, and, therefore, as an important indicator for autophagic flux [27,28]. Collectively, these results suggested during $CoCl_2$-induced leukemic cell differentiation, autophagosome formation increased while the degradation of autophagic vacuoles (AVs) decreased.

3.2. CQ enhances $CoCl_2$-induced growth arrest and differentiation of NB4 cells

Based on the observation that $CoCl_2$ increases autophagosome formation without enhancing the degradation of p62 protein, this effect was similar to that of CQ as a lysosomotropic agent with the impairment of autophagic vesicle clearance and the increase in the accumulation of AVs [22]. Thus, CQ was introduced to test whether autophagy deficiency (especially the defect of late step in autophagy) affects $CoCl_2$-induced differentiation. We first tested the inhibitory effect of CQ on autophagic activity when $CoCl_2$ was treated together. To this end, NB4 cells were treated with 50 μM $CoCl_2$ with and without 10 or 20 μM of CQ, either two concentrations of the latter having the inhibitory effect on autophagy commonly used in different cell lines [24], for 6 days. As depicted in Fig. 2A, CQ treated alone significantly increased LC3-II protein levels in a concentration-dependent manner in NB4 cells. When CQ (10 or 20 μM) was used with 50 μM $CoCl_2$ in combination, the accumulation of LC3-II protein was further enhanced compared to those treated with the either concentration of CQ or with $CoCl_2$ treatment alone. These results indicated CQ could efficiently suppress the basal level of autophagy and $CoCl_2$-induced alteration of autophagic flux [22]. Notably, CQ significantly enhanced $CoCl_2$-induced LC3-II accumulation, indicating that $CoCl_2$ is not likely to suppress autophagic flux at the maturation and degradation stage [24]. We then determined whether CQ influenced the growth inhibition induced by 50 μM of $CoCl_2$ for the time course. As shown in Fig. 2B, the treatment of $CoCl_2$ combined with both concentrations of CQ induced significantly growth inhibition than the treatment of $CoCl_2$ alone although the treatment of 20 μM CQ also produced a time-dependent growth arrest of NB4 cells. Moreover, treatment with CQ or $CoCl_2$ at the indicated concentrations, failed to reduce the cell viability, and their combination also kept cell viability over 70% (Table 1).

We next investigated possible effects of the combined treatment of $CoCl_2$ with CQ on the differentiation of NB4 cells. In agreement with our previous reports [5,29], treatment of NB4 cells with 50 μM $CoCl_2$ for 6 days exhibited mature-related morphological alteration, such as condensed chromatin and a decreased nuclei/cytoplasm ratio with smaller nuclei (Fig. 2C). Treatment of 50 μM $CoCl_2$ also increased $CD11b^+$/$CD11c^+$ cells but failed to increase NBT-positive cells (Fig. 2D and F). Interestingly, the cells treated with $CoCl_2$ plus both concentrations of CQ displayed morphologically more mature myeloid cells (Fig. 2C) with significantly increased $CD11b^+$ and $CD11c^+$ expression (Fig. 2D), compared to that treated with $CoCl_2$ alone. However, the combined treatment of $CoCl_2$ and CQ did not show the positive NBT reaction (Fig. 2F), one of the markers of mature granulocytes. We also measured mRNA levels of NCF-1 and Spp-1 genes by real-time quantitative PCR, since these genes have been used as leukemic cell differentiation signatures [30]. As shown in Fig. 2E, the NCF-1 and Spp-1 mRNA levels were significantly higher in NB4 cells treated with $CoCl_2$ plus CQ than treated with $CoCl_2$ alone. Treatment of CQ alone with both concentrations did not affect the expression of CD11 and of NCF-1 and Spp-1 mRNA. Overall, these results indicated that CQ potentiated $CoCl_2$-induced growth arrest and differentiation of NB4 cells.

3.3. CQ increases $CoCl_2$-induced HIF-1 α accumulation of NB4 cells

To further investigate the potential mechanism of CQ-enhanced differentiation induction by $CoCl_2$, we examined the expression levels of several differentiation-related transcriptional factors that are important for hematopoietic cell differentiation as well as HIF-1

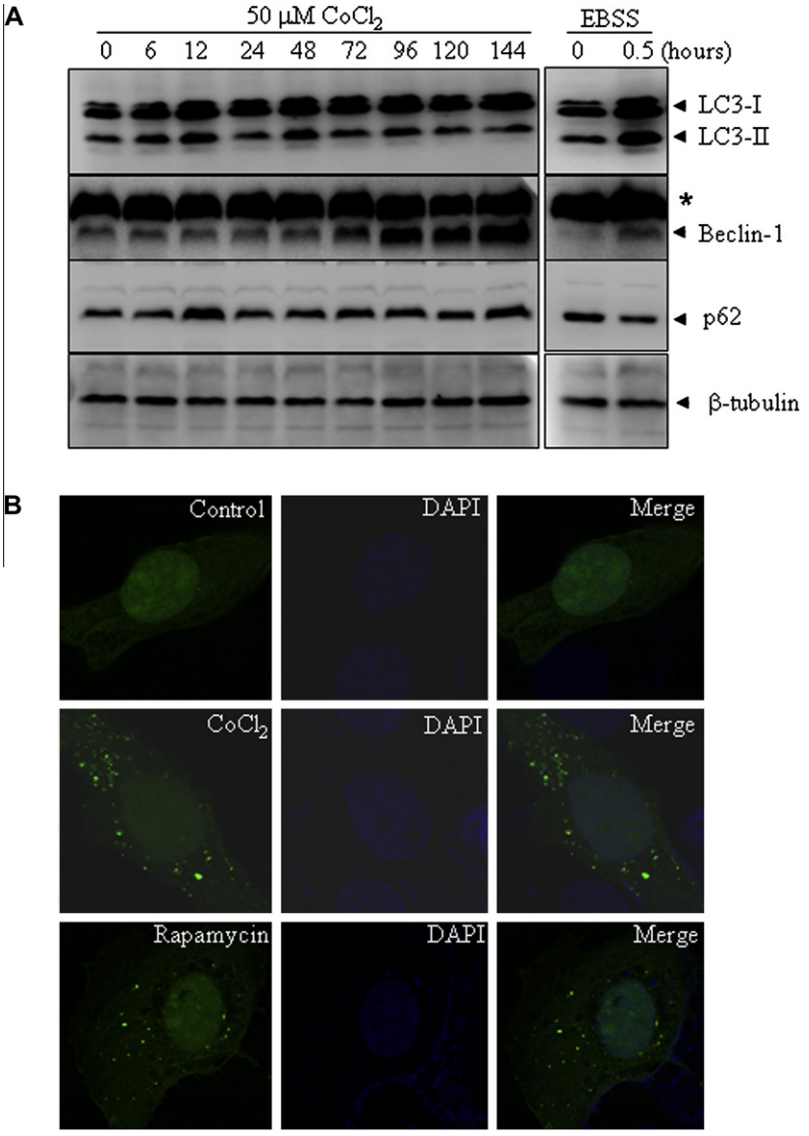

Fig. 1. The effect of nontoxic concentration of $CoCl_2$ on autophagic activity during differentiation of NB4 cells and U_2OS. (A) NB4 cells were treated with or without 50 μM $CoCl_2$, or with EBSS for the indicated hours. Cell lysates were harvested for immunoblotting proteins as indicated. (B) U_2OS cells were transiently transfected with GFP-LC3 for 24 h, followed by treatment with or without 50 μM $CoCl_2$, or 0.5 μM rapamycin for 6 h. Then the cells were observed by confocal microscopy. The representative images of GFP-LC3-transfected cells with the indicated treatments were shown.

α, the latter having been identified as a crucial regulator of hypoxia-induced leukemic cell differentiation [5,8,10,12]. Consistent with our previous reports [5,10], treatment of $CoCl_2$ (50 μM) increased HIF-1 α protein, but not HIF-1β and three differentiation-related transcriptional factors (PU.1, C/EBP α and Runx1), as shown in Fig. 2G. Unlike $CoCl_2$, treatment with CQ (10 or 20 μM) alone for 6 days could not affect HIF-1 α protein level, but it could significantly potentiate $CoCl_2$-induced increase in HIF-1 α (Fig. 2G). Of note, the effect of CQ at 10 μM on $CoCl_2$-induced HIF-1 α accumulation was equivalent to that of 20 μM CQ. HIF-1 β protein levels remained stable in the NB4 cells regardless of the treatment regimes used. On the other hand, the combined treatment of $CoCl_2$ and CQ did not influence the expression levels of PU.1, C/EBP α and Runx1 protein.

3.4. CQ also enhances $CoCl_2$-induced growth arrest and differentiation in U937 cells

In order to determine whether the combined effects of $CoCl_2$ with CQ are present in other AML subtypes, we examined the effects of $CoCl_2$ plus CQ on non-APL monocytic U937 cells. As reported previously [5,29], treatment with $CoCl_2$ at 50 μM for 6 days produced significant growth arrest and differentiation induction in U937 cells (Fig. 3A–D). Like what occurred in NB4 cells, 20 μM CQ also enhanced $CoCl_2$-induced growth arrest (Fig. 3A and B) and differentiation in U937 cells, as determined by the alteration of cell morphology with mature characters (Fig. 3C) and the increases in percentages of $CD11b^+/CD11c^+$ cells and expression of NCF-1 and Spp-1 genes (Fig. 3D). Similarly, CQ also enhanced $CoCl_2$-induced HIF-1 α protein accumulation in U937 cells (Fig. 3E).

3.5. Suppression of both LAMP1 and LAMP2 expression by shRNAs potentiates $CoCl_2$-induced differentiation of U937 cells

Lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2), two major components of lysosomal membrane proteins, have been shown to be required for the proper fusion of autophagosome and lysosome *in vivo* and *in vitro* [31,32]. To further address whether CQ acts as an autophagy inhibitor to increase $CoCl_2$-induced cell differentiation, suppression of LAMP1 and

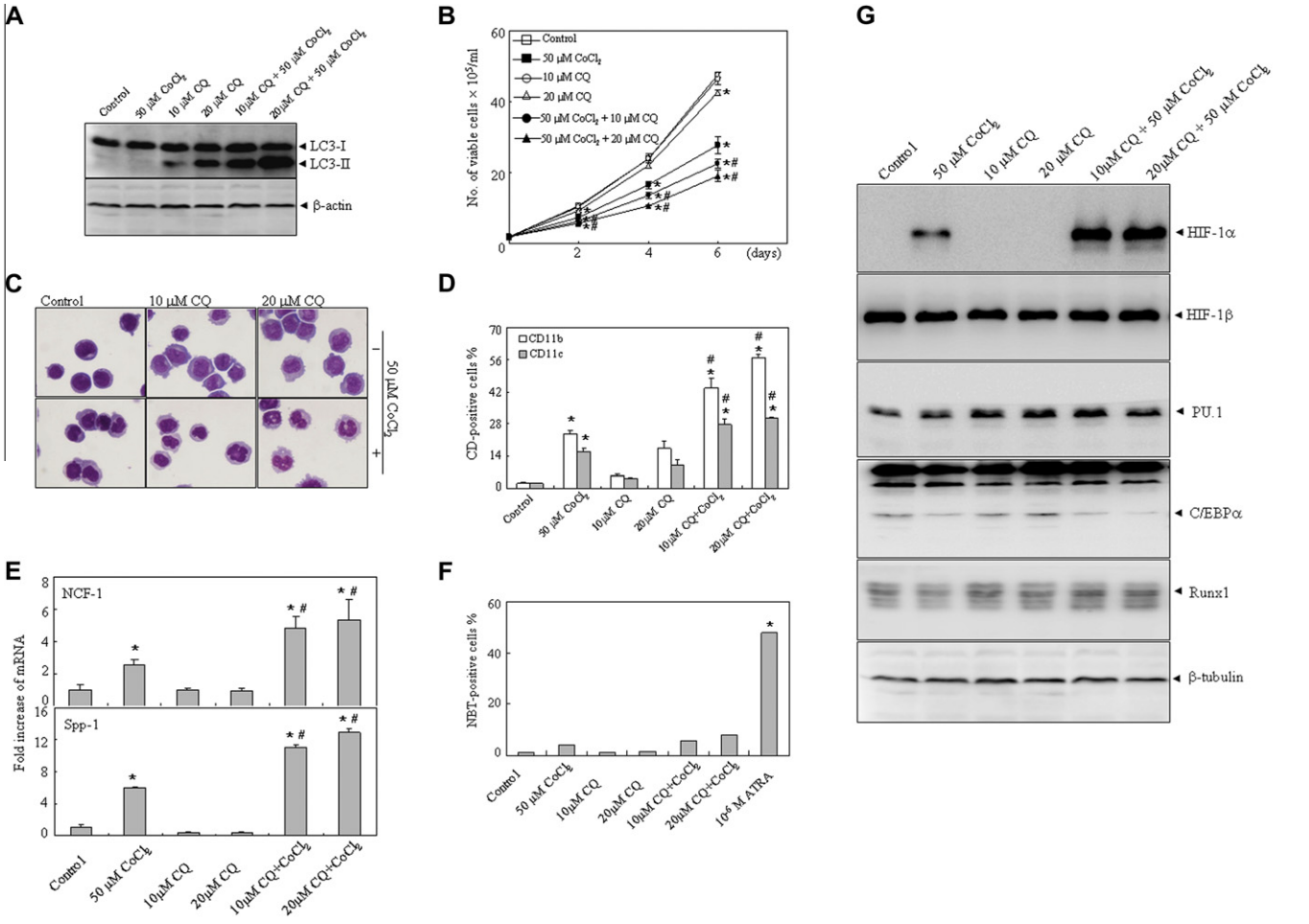

Fig. 2. Effects of $CoCl_2$, CQ and their combined treatment on LC3 conversion, cell growth, differentiation and hypoxia-related proteins of NB4 cells. NB4 cells were treated with the indicated regimes for 6 days (A, C, E–G), 4 days (D) or the indicated days (B). (A, G) LC3 protein and the indicated proteins were analyzed by western blot with β-actin or β-tubulin as equally loading control. (B) Viable cell numbers were measured with trypan-blue exclusion assay. (C) Cell morphology was observed under microscope following Wright's staining of cells that were collected onto slides by cytospin. (D) The indicated CD-positive cells were analyzed by flow cytometry. (E) Real-time quantitative PCR assay was performed to examine the expression levels of NCF-1 and Spp-1 mRNA. (F) NBT-positive cells were analyzed. NB4 cells were treated with 10^{-6} M ATRA for 2 days as a positive control. The symbols ∗ and # respectively present p values of less than 0.05 compared with the untreated cells and the cells treated with $CoCl_2$ alone.

Table 1
Effects of the indicated regimes on survival of NB4.

Treatment for 6 days	Cell viability (mean ± SD%)
Cont	98.6 ± 0.6
50 mM $CoCl_2$	93.2 ± 1.4
10 mM CQ	95.9 ± 1.4
20 mM CQ	95.5 ± 0.7
10 mM CQ + 50 mM $CoCl_2$	83.5 ± 3.2
20 mM CQ + 50 mM $CoCl_2$	73.0 ± 2.6

LAMP2 expression can mimic the action of CQ resulting in the blockage of autophagosome fusion and degradation. For this purpose, U937 cells were transfected by two pairs of shRNA specifically targeting LAMP1 (shR-L1-1 and shR-L1-4) and a negative control (NC) shRNA. As shown in Fig. 4A, shR-L1-1 and shR-L1-4, but not NC, significantly eliminated endogenous LAMP1 protein. The similar knock down effect of LAMP2 was also produced in U937 transfected by shR-L2-1 and shR-L2-3, two pairs of shRNA against LAMP2. Under the interference of these specific shRNAs, the differentiation of U937 induced by 50 μM of $CoCl_2$ did not change (Fig. 4B) although elimination of LAMP1 by shR-L1-4 displayed a decreased response to $CoCl_2$ incubation. Several lines of evidence suggest that the functions of LAMP1 and LAMP2 are overlapped, due to the mice deficient of both LAMP1 and LAMP2, but not either one of them, leads to the disruption of the autophagosome maturation and embryonic lethal phenotype [31,32]. Therefore, we further tested whether the suppression of both LAMP1 and LAMP2 expression affects the differentiation effect induced by $CoCl_2$. To this end, U937 cells stably expressing shR-L1-1, as the parental cells, were further transfected with two pairs of GFP-tagged shRNAs targeting LAMP2 (shR-L2-1 and shR-L2-3) and NC. After 72 h, the cells with second transfection were sorted to obtain >90% GFP-positive cells that express GFP-tagged shRNAs. As depicted in Fig. 4C, shR-L1-1/L2-1 and shR-L1-1/L2-3, but not shR-L1-1/NC, completely suppressed LAMP2 protein expression in the U937/shR-L1-1 cells. The cells were then treated with or without 50 μM $CoCl_2$ for 6 days. As assessed by the percentage of $CD11b^+/CD11c^+$ cells, intriguingly, $CoCl_2$-induced differentiation was significantly increased in the silencing of expression of both LAMP1 and LAMP2 proteins of U937/shR-L1-1/shR-L2-1 and U937/shR-L1-1/shR-L2-3 cells, compared with that in the silencing of LAMP1 expression of U937/shR-L1-1/NC cells (Fig. 4D), suggesting that CQ was capable of sensitizing $CoCl_2$-induced cell differentiation through the inhibition of autophagy at the late stage.

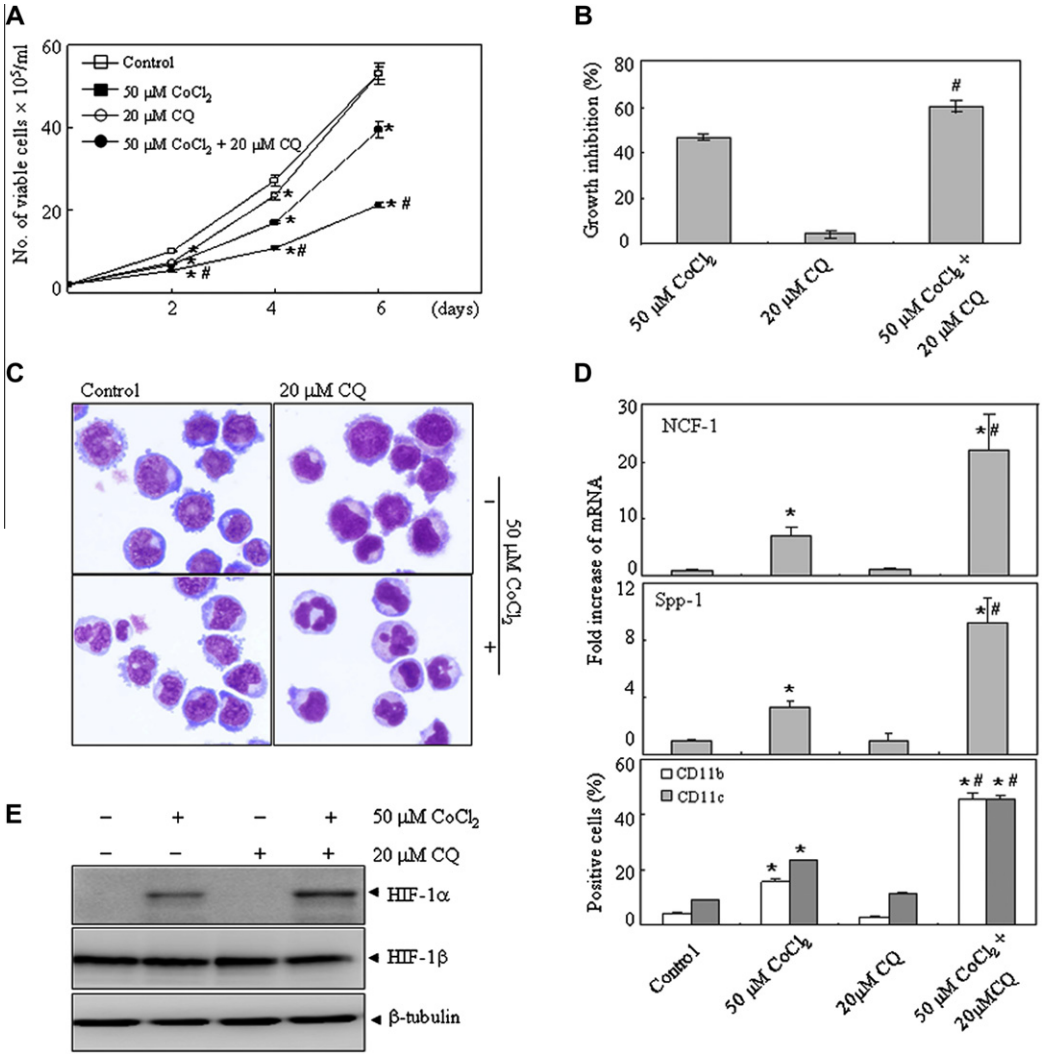

Fig. 3. Effects of CQ on $CoCl_2$-induced growth arrest, cell differentiation and HIF-1 α expression in U937 cells. The cells were treated with and without 50 μM $CoCl_2$, 20 μM CQ alone and in combination for 6 days. (A) Viable cells were measured for the indicated days. (B) The growth inhibition % with up to 90% viable cells was analyzed by the data of 6 days from (A). (C) Cell morphology was observed under microscope. (D) Real-time quantitative PCR was performed to measure the expression of the indicated genes and $CD11b^+/CD11c^+$ cells were analyzed by flow cytometry. (E) The indicated proteins were analyzed by western blot with β-tubulin as a loading control. The symbols ∗ and # respectively present *p* values of less than 0.05 compared with the untreated cells and the cells treated with $CoCl_2$ alone.

4. Discussion

Previously, Sasaki et al. [33] reported that CQ induces APL cell line HL-60 to differentiate into basophils. CQ has also been found to significantly induce differentiation and growth arrest of K562 cells with producing fetal hemoglobin by targeting polyamine pathway [34]. In the present study, we demonstrated that non-toxic concentrations of CQ increased $CoCl_2$-induced growth arrest and cell differentiation of AML cell lines in a subtype-independent manner, as evidenced by morphologic criteria, myeloid differentiation-related antigens and the marker genes NCF-1 and Spp-1. These results suggest that CQ can modulate $CoCl_2$-induced cell differentiation.

CQ exerts a pleiotropic action such as increasing vacuolar pH through being trapped in acidic organelles including lysosomes. Disrupting lysosomal acidification thus results in the impairment of autophagosome fusion and degradation [22,24]. To validate whether the sensitizing effect of CQ depends on its ability to inhibit autophagy, we tested the effect of Beclin-1 suppression on $CoCl_2$-induced cell differentiation. Unexpectedly, knockdown of Beclin-1 with shRNAs did not affect $CoCl_2$-induced differentiation (data not shown), suggesting that autophagy might not be involved in $CoCl_2$-induced differentiation or $CoCl_2$-induced autophagy might be in a Beclin-1-independent manner. However, we found that interference of the expression of both LAMP1 and 2, mimicking the inhibitory effect of CQ on autophagy at the late phase, could reproduce the increased sensitization effect of $CoCl_2$-induced differentiation. Therefore, CQ-enhanced $CoCl_2$-induced differentiation could be conducted through inhibiting autophagy. No response to sensitizing $CoCl_2$-induced differentiation, which was observed in knockdown of Beclin-1 leukemic cells, was likely due to the latter possibility. Indeed, recently several studies have shown that autophagy can occur in a Beclin-1-independent way [35,36].

Accumulating evidence showed that hypoxia induces autophagy dependent and/or independent of BNIP3 protein, an atypical BH3-only protein induced by HIF-1 [37]. Our results showed that $CoCl_2$ altered the autophagic activity in a manner of substantial accumulation of autophagosomes without an increase in the degradation of AVs, as assessed by the increase of forming GFP-LC3-punctate structures, the elevation of LC3-II and Beclin-1 protein levels as well as no significant alteration of p62 protein. p62, targeting cargoes for degradation through autophagy [38], has also been found to be degraded through autophagy in response to

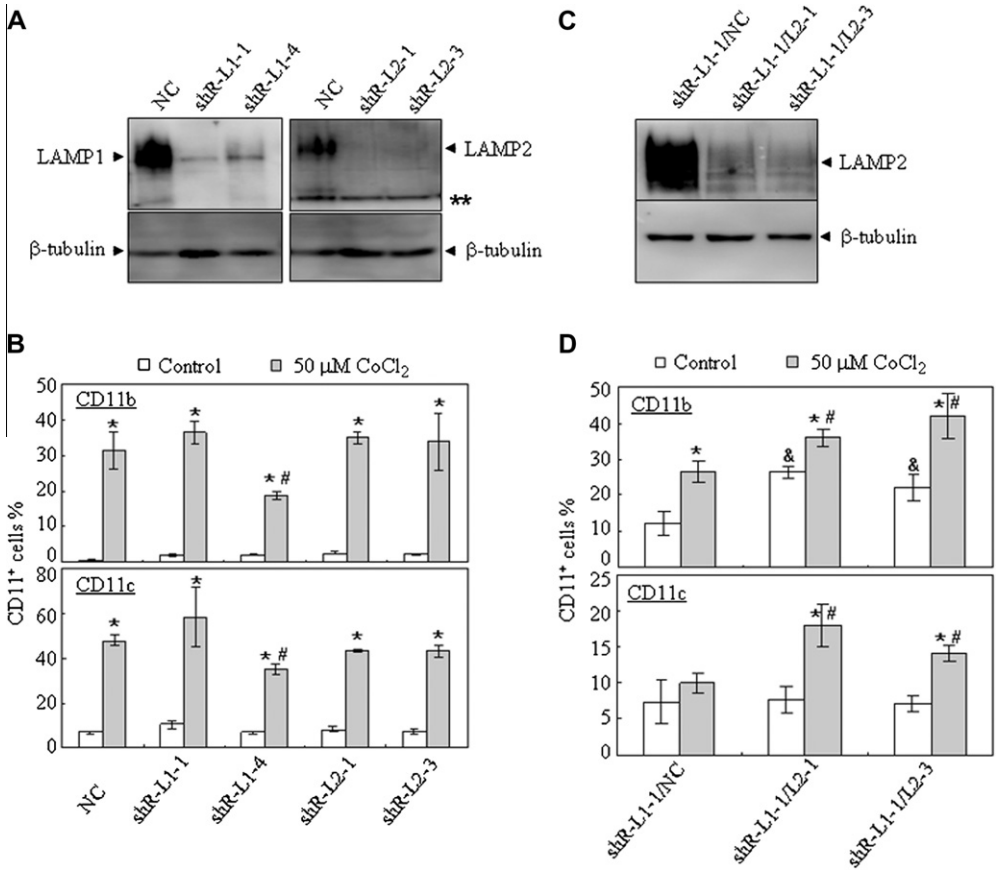

Fig. 4. Effects of the suppression of LAMP1 and/or LAMP2 expression by shRNAs on $CoCl_2$-triggered differentiation of U937 cells. (A and B) U937 cells were stably transfected into the indicated shRNAs or NC. Then the cells were treated with or without 50 μM $CoCl_2$ for 6 days. The effect of LAMP1 or LAMP2 knock down was analyzed by western blot (A). The symbol ** pointed to a nonspecific band. (C and D) shR-L1-1-expressing U937 cells were further transfected into GFP-tagged shR-L2-1, shR-L2-3 or NC. After 72 h, GFP-positive cells were sorted by BD FACSAriaII cell sorter. The cells collected after sorting were then treated with and without 50 μM $CoCl_2$ for 6 days. The suppression of LAMP2 protein in those collected cells was examined by western blot with β-tubulin as a loading control (C). Percentages of $CD11b^+/CD11c^+$ cells were analyzed by cytometry (B and D). The symbols * and # respectively present *p* values of less than 0.05 compared with the corresponding untreated cells expressing different shRNAs and NC cells treated with $CoCl_2$ (B) or shR-L1-1/NC cells treated with $CoCl_2$ (D). Symbol & stands for *p* value of less than 0.05 compared with the untreated shR-L1-1/NC cells (D).

hypoxia but independent of HIF-1 [39]. However, we found that $CoCl_2$-triggered autophagy alteration was not accompanied by the decrease of p62. This discrepancy was possibly due to the different effects of real hypoxia and $CoCl_2$. It is a paradox with the notion that autophagy activity increases during differentiation, but the fact is that CQ and interference of the expression of LAMP1 and LAMP2 potentiate $CoCl_2$-induced differentiation, implicating the inhibition of autophagy at its late phase might be necessary for leukemic cell differentiation induced by $CoCl_2$. We extrapolated that the contradiction was possibly due to the dynamic autophagy process during cell differentiation.

The results showed that CQ enhanced $CoCl_2$-induced accumulation of HIF-1 α protein and cell differentiation in NB4 and U937 cells, while CQ did not increase 1% O_2-induced HIF-1 α and differentiation (Supp. Fig. S1), providing further evidence that HIF-1 α protein plays an essential role in leukemic cell differentiation. CQ-potentiated HIF-1 α protein accumulation was possibly mediated by interfering in the proteosomal digestion pathway because autophagy inhibition compromises degradation of ubiquitin–proteasome pathway substrates [40]. The reason for different effects of CQ on $CoCl_2$- and 1% O_2-induced HIF-1 α protein accumulation is not clear.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.052.

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