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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

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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differentiation induced by ATRA and VitD3 [18,20,21]. Here we 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 FACSCalibur). 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-

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