Role of CDKN2A and CDKN2B in ATRA-induced autophagy and differentiation of acute promyelocytic leukemia cells

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1. Abstract

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML), which leads to a differentiation block at the promyelocytic stage, due to the expression of the oncoprotein PML-RARA. APL is successfully treated with targeted therapy in the clinic using all-trans retinoic acid (ATRA), which activates cell differentiation. It is known that autophagy is crucial in APL. However, the exact mechanisms are still poorly understood. Reports on CDKN2A and CDKN2B genes, which encode tumor suppressor proteins, suggest that they might play a role in ATRA-induced autophagy. The genes are known to be hypermethylated in leukemia and their expression is impaired. Since they potentially play a key role in therapy response, they were analyzed in this diploma thesis. By knocking down CDKN2A and CDKN2B in APL cells, no significant difference in differentiation has been observed by morphology or expression of differentiation markers in ATRA-treated cells compared to untreated cells. By analyzing autophagy markers, CDKN2B seemed to have an impact on ATRA-induced autophagy, while CDKN2A did not show any tendency whatsoever. Therefore, the role of CDKN2B in autophagy should be analyzed with further experiments to find out exactly how it affects autophagy in ATRA-treated APL.

2. Introduction

More than 97% of the APL cases express the translocation t(15;17). Without treatment, APL is one of the most fatal forms of acute leukemia, due to its characteristic bleeding diathesis due to fibrinogenopenia and disseminated intravascular coagulation [1].

ATRA belongs to the family of retinoids and is a metabolite of vitamin A, which is able to bind the PML-RARA gene at the retinoic acid-binding domain of RARA mosty, resulting in a change of conformation. This conformational change leads to a release of transcriptional co-repressors and recruitment of transcriptional co-activators, shown in figure 2.1, activating transcription of RARA target genes and initiating differentiation. On the other hand, the destabilization of PML-RARA also induces its degradation via the proteasome and autophagy [2].

CDKN2A and CDKN2B genes (also known as the INK4a/ARF/INK4b locus) are located on the human chromosome 9p21 as shown in figure 2.2. They encode the cell-cycle regulators p16INK4a (CDKN2A) and p15INK4b (CDKN2B) mainly described as tumor suppressor proteins [3].

This work aims at dissecting the autophagy response mechanism and that might help to design a better therapeutic strategy by targeting autophagy.

3. Aims/ Leading Question

The aim of this project is, to find out what effects CDKN2A and CDKN2B have on ATRA-induced autophagy and differentiation, since it has been shown that CDKN2A binds to ATG5 and furthermore, that ATG5 is crucial for APL differentiation therapy upon ATRA-induced autophagy, while CDKN2A did not have an impact on ATRA-induced autophagy. The LC3B expression of NB4 cells in shown in figure 5.3. The samples with added Bafilomycin show increased levels. Interestingly, NB4 shCDKN2B cells have a lower LC3B level upon treatment with ATRA and bafilomycin.

4. Material, Methods and Approach

First, lentivirus were generated to deliver small hairpin (sh-)RNAs targeting either CDKN2A or CDKN2B or a non-targeting shRNA (SHC002) in NB4 and HL60 AML cells. Knockdown efficiency was then measured by Western Blot or quantitative polymerase chain reaction (qPCR)/Taqman. Next, knockdown cells were treated with ATRA for two and four days to induce granulocytic differentiation. Differentiation was assessed by CD11b cell surface expression using FACS, CEBPE and CSF3R using qPCR/Taqman. In parallel, autophagy activity was measured via western blot of ATG5-ATG12 complex formation and LC3B lipidation and via immunofluorescence microscopy by monitoring the LC3B dots formation. Unfortunately, not all of the experiments were repeated, due to time issues.

5. Results

Figure 5.1 shows the TaqMan analysis of CDKN2B mRNA levels in HL60 cells after two and four days upon ATRA. In SHC002 CDKN2B levels are lower after two days treatment with ATRA and is going back to steady state levels after four days ATRA treatment. Furthermore, shCDKN2B 575 and 579 have less CDKN2B expression compared to SHC002 control cells.

The FACS analysis in figure 5.2 shows the median-fluorescence-intensity (MFI) of CD11b surface expression in NB4 and HL60 cells after two and four days upon ATRA treatment. In the NB4 cells, shCDKN2B 575 express significantly more CD11b at steady-state condition compared to SHC002 control cells. Upon ATRA treatment, shCDKN2B 567 and 575 express significantly less CD11b after 4 days. In HL60, shCDKN2B 579 cells have a reduced CD11b level after two days of ATRA treatment.

6. Discussion

CDKN2A

Unfortunately I was not able to detect a knockdown by Western Blot. Therefore, I conclude that the shRNAs 950, 971 and 1129 do not provide good inhibition of CDKN2A expression in either NB4 nor HL60 cells, thus other shRNA would need to be tested to find a more efficient one. AOD for TaqMan was not provided. Due to these circumstances, the following experiments are not convincing.

CDKN2B

I could confirm knockdown efficiency in HL60 cells at the mRNA level, as it is apparent in figure 5.1. Indeed, the expression levels are lower in shCDKN2B cells, compared to the control cells SHC002. The fact that I could not detect CDKN2B in NB4 cells while I could in HL60 leads me to the conclusion that CDKN2B levels vary within AML cell lines.

Considering the FACS results of the differentiation marker CD11b in figure 5.2, CDKN2B seems to have no impact on differentiation. Actually, CDKN2B knockdown cells showed no tendency to differentiate more or less, as it would have been apparent in increased or reduced expression in the bar charts. Morphological and Taqman results also showed no drastic difference compared to the control cells.

CDKN2B seems to have an impact on autophagy, as the autophagic flux upon ATRA treatment is reduced in NB4 shCDKN2B as shown in WB in ATRA + Baf treated cells (figure 5.3), as they accumulate less LC3B compared to SHC002 control cells. Since CDKN2B inhibition seems to have an impact on autophagy, several additional experiments need to be performed to confirm our preliminary data. Importantly, detection of LC3B dots formation by IF has to be performed in presence or absence of CDKN2B.

To demonstrate the effect of CDKN2A in ATRA therapy other shRNA would need to be tested. In addition, using AOD detecting CDKN2A will give us another measurement for KD efficiency at the mRNA level.

References


Figures

Fig. 2.1 Ablain, J. and de Thié, H. (2011) Revisiting the differentiation paradigm in acute promyelocytic leukemia | Journal of Hematological & Oncological Treatment 12:6.
Fig. 2.2 Ablain, J. and de Thié, H. (2011) Revisiting the differentiation paradigm in acute promyelocytic leukemia | Clin Cancer Res, 15(20)