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ORIGINAL ARTICLE

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Decitabine activates specific caspases downstream of p73 in myeloid leukemia

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Abstract The demethylating effect of 5-aza-2′ deoxycytidine (decitabine, DAC) has been well characterized. The molecular events downstream of methylation inhibition are less well known. Here, DAC was shown to induce apoptosis in acute myeloid leukemia (AML) cells (p53 mutant and wild type) but not in epithelial or normal peripheral blood mononuclear cells. Apoptosis was characterized by activation of the mitochondrial but not the receptor death pathway, as demonstrated by the release of cytochrome *c* and loss of mitochondrial membrane potential. Western blotting and enzyme assays showed that caspase-3, but not caspase-6 or caspase-8, were activated. Decitabine induced expression of the cell cycle inhibitor p21, arresting AML cell lines in G1 of the cell cycle. Expression of p21 was induced irrespective of the methylation status of its promoter, mediated instead via reexpression of the tumor suppressor p73, an upstream regulator of p21. The promoter of p73 was hypermethylated in AML cell lines in vitro and in primary AML cells ex vivo but not in DAC-resistant epithelial cells. In conclusion, DAC acts on leukemic myeloid cells via caspase activation, which may be dependent on demethylation of the hypermethylated p73 promoter and consequent reexpression of p73.

Keywords Acute myeloid leukemia (AML) · Apoptosis · Hypermethylation · Caspases · p73 · p21

Introduction

DNA hypermethylation is an important epigenetic mechanism that can silence leukemia-suppressing genes and play a crucial role in leukemogenesis [11]. During DNA replication, DNA methyltransferases (DNMTs) cause methylation of CpG islands in the 5′ region of genes, resulting in the inhibition of transcription [17]. 5-aza-2′deoxycytidine (decitabine, DAC) is a nucleoside analog that is integrated into DNA and inhibits DNA methylation by the trapping of DNMTs. Several clinical studies have reported promising therapeutic activity of DAC in patients with myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML), and acute myeloid leukemia (AML) [10, 12, 22]. Decitabine effectively switches off methylation-mediated gene repression, as seen for p15INK4b and other genes implicated in AML [3].

Decitabine-induced effects occurring downstream of methylation sites are less well characterized. In our study, we have investigated the connection between DNA methylation inhibition of DAC and its ability to induce apoptosis. To establish the molecular rationale for drug action, we have explored which DAC-inducible, methylation-dependent genes mediate the action of the drug in AML cells.

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Materials and methods

The experimental conditions used in this paper have been previously described in Schmelz et al. [19]. However, some details of the specific conditions and materials are described below.

Cell culture and DAC incubations

The human AML cell lines OCI-AML2, CTV-1, EOL1, KG1, and HL60, the histiocytic lymphoma cell line U937, and the epithelial cell lines 293 and HeLa were obtained from the Deutsche Sammlung fuer Mikroorganismen und Zellen (Braunschweig, Germany). Cells were cultured as described elsewhere [19]. Peripheral blood mononuclear cells (PBMCs) from healthy donors and primary AML blasts (peripheral blood or bone marrow) were isolated using Ficoll gradient centrifuga-

tion (Biochrome, Berlin, Germany). For DNA methylation analysis, primary cells from patients with newly diagnosed and untreated AML of FAB subtypes M0, M1, M4, and M5 were examined. Cells were exposed to a single dose of 0.1–10 μM DAC (Sigma, Taufkirchen, Germany) in a phosphate-buffered solution (PBS). Decitabine aliquots were stored at −20°C.

RNA preparation and reverse transcriptase–PCR

Total RNA was isolated using a Trizol reagent (Invitrogen, Karlsruhe, Germany) and cDNA-synthesized [19]. The primer sequences for p73, p21, and glyceraldehydephosphate dehydrogenase (GAPDH) and polymerase chain reaction (PCR) conditions were used as described previously [19].

Methylation-specific PCR and *Hpa*II digestion of genomic DNA

Methylation-specific PCR (MSP) was undertaken using bisulfite-modified genomic DNA as template and p73 methylation-specific primers [5′-GGACGTAGCGAAATCGG GGTTC-3′ (position 875907–875928) and 5′-ACCCCG AACATCGACGTCCG-3′ (position 875947–875966)], resulting in the amplification of CpG-methylated sequences of p73 (accession number NT_004321). Amplification products were separated via an 8% nondenaturing polyacrylamide gel and visualized by silver staining.

To examine CpG methylation in the p21 promoter region, genomic DNA was digested with 10 U of the methylation-sensitive restriction enzyme *Hpa*II overnight. 250 ng digested and nondigested (control) genomic DNA were used as template for PCR amplification [19].

Detection of apoptosis and measurement of caspase activity

DNA fragmentation and cell cycle analysis were carried out as described [19]. Data are presented as percentage of hypodiploid cells, i.e., cells in sub-G1, reflecting the number of apoptotic cells. For cell cycle analysis, cellular DNA content was quantified after linear amplification using CELLQuest software.

Apoptosis was also detected by staining the cells with annexin–fluorescein isothiocyanate (FITC)/propidium iodide and subsequent fluorescence-activated cell sorter (FACS) analysis.

Caspase-3 activity was assayed in cytosolic extracts [100-μg samples in 5 mM dithiothrietol (DTT)] using a photometric assay. The release of *p*-nitroanilide (*p*NA) from peptides containing the sequence DEVD (Calbiochem, Bad Soden, Germany) was measured using an automated plate reader (SLT Labinstruments, Crailsheim, Germany). Preparation of protein lysates and immunoblotting was performed as described [19].

Results

Decitabine-induced apoptosis

Decitabine-induced apoptosis was examined in the AML cell lines CTV-1, EOL, OCI-AML2, KG1, HL60, and the histiocytic lymphoma cell line U937. Using DNA fragmentation, apoptosis could be detected after 16, 48, 72, and 96 h following treatment with a single dose of 1 or 5 μM DAC. Decitabine induced apoptosis in a time- and dose-dependent manner in all leukemic myeloid cell lines tested. Treatment with 1 μM DAC for 96 h resulted in 20.5% (CTV), 48.7% (EOL), 37.6% (OCI-AML2), 21.2% (KG1), 12.5% (HL60), and 4.3% (U937) DNA fragmentation. These results were confirmed by annexin–FITC/PI FACS analysis. In contrast, apoptosis was absent in the epithelial cell lines 293 and HeLa after treatment with up to 10 μM DAC for 96 h (as a single dose). Decitabine (72 h incubation) was much less cytotoxic to PBMCs from healthy donors compared with AML patients (data not shown). Thus, we found that DAC specifically induced apoptosis in AML but not in epithelial cell lines.

To analyze the use of different apoptosis pathways, the activation of specific caspases was measured following treatment with DAC. Caspase-3 activity was seen in EOL and CTV cells 17 and 39 h after treatment, respectively. Significant caspase-3 activity was detectable in HL60 and KG1 cells 72 h after treatment. In contrast, caspase-3 activity was absent in OCI-AML2 and U937 cells (data not shown). Results were confirmed by Western blotting to detect the active large subunit of caspase-3. Activation of caspase-3 after exposure to 5 μM DAC was detectable earlier in EOL and CTV-1 cells (Fig. 1a) than in KG1 and HL60 cells (Fig. 1b). Low levels of caspase-3 processing were seen in U937 cells but not in OCI-AML2 cells (Fig. 1b).

In contrast, treatment of AML cell lines with DAC did not induce processing of effector caspase-6 as demonstrated by Western blot (Fig. 1a,b). Caspase-8 activation was not seen (Fig. 1a,b), indicating that the receptor-mediated apoptosis pathway might not be involved in the action of DAC.

Release of cytochrome *c* and mitochondrial membrane potential

The mechanism of DAC-induced apoptosis was further investigated through analysis of the mitochondrial apoptosis pathway. Western blotting analysis of cytosolic extracts showed that cytochrome *c* was already released after 2 days of drug exposure in cell line EOL, which is most sensitive to DAC. In cell lines KG1, OCI-AML2, CTV-1 and HL60 release of cytosolic cytochrome *c* was only detectable after 3 days of treatment with DAC (Fig. 1c).

Mitochondrial membrane potential dissipation ($\Delta\Psi_m$) represents another crucial signal for the induction of apoptosis via the mitochondrial pathway [8]. Decitabine (5 μM) caused a loss of $\Delta\Psi_m$ in AML cells (data not

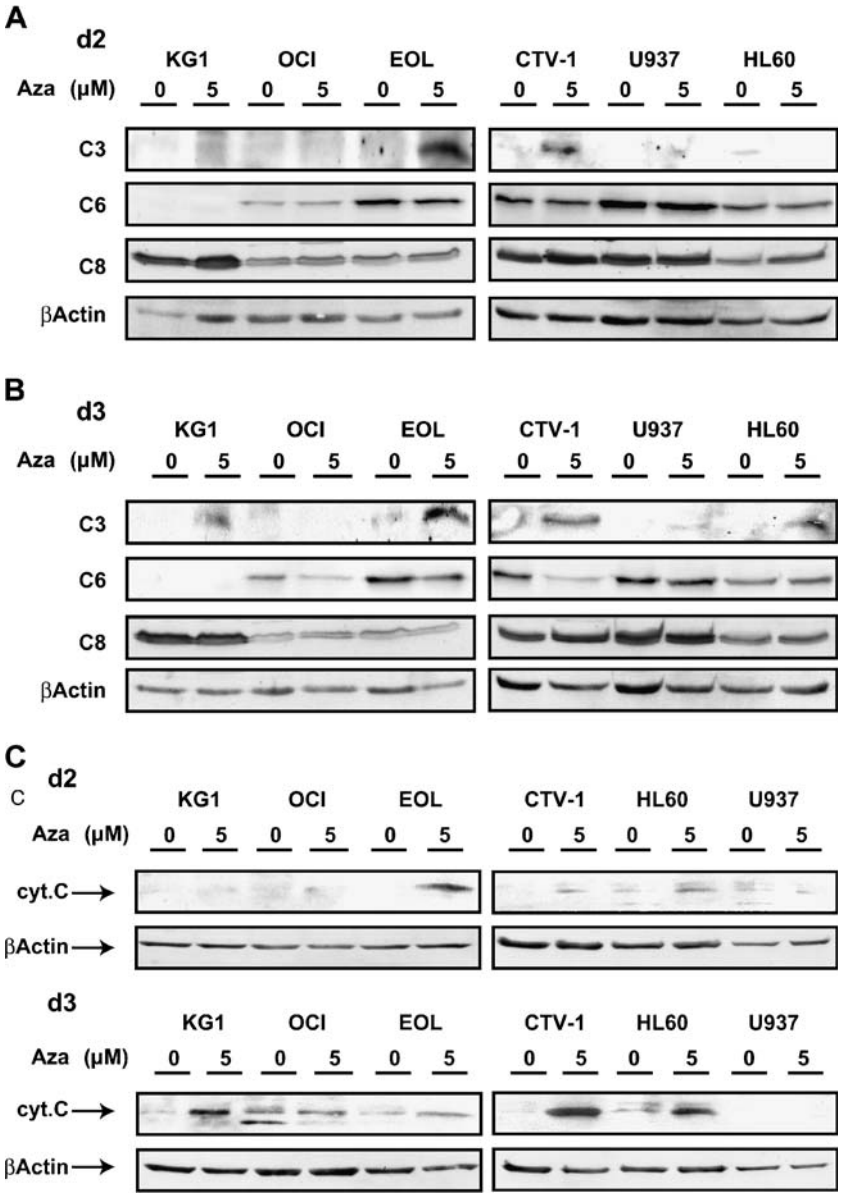

Fig. 1 Representative Western blot analysis of lysates from AML cell lines exposed to 5 μM decitabine or medium for 2 days (**a**) or 3 days (**b**). Immunostaining was performed subsequently with antibodies for the active, large subunit (p18) of caspase-3, caspase-6 zymogen, and caspase-8 zymogen. β-actin served as control. (**c**) Release of cytochrome *c* from mitochondria to the cytosol as assessed by Western blot analysis following treatment with 5 μM decitabine for 2 or 3 days using an antibody against cytochrome *c*. β-actin served as control. Two additional experiments yielded similar results. Reprinted with permission from Wiley and Sons [19]

shown). Thus, we could detect activation of the mitochondrial apoptosis pathway by DAC, as evidenced by the release of cytochrome *c* to the cytosol and a loss of the mitochondrial membrane potential, $\Delta\Psi_m$.

Cell cycle arrest

Observations during DNA fragmentation experiments suggested that induction of apoptosis in DAC-treated cells is associated with an arrest of cells in different phases of the cell cycle. Decitabine treatment resulted in arrest in the G1 phase of the cell cycle in OCI-AML2 but not KG1 cells, although both cell lines displayed similar amounts of apoptosis (data not shown).

Cell cycle arrest in G1 has been attributed to the induction of the cell cycle regulator p21/WAF [4]. p21 expression was therefore assessed using Western blotting. Decitabine increased the expression of p21 in G1-arrested OCI-AML2 cells. In contrast, KG1 cells that were not arrested in G1 did not express p21 protein (data not shown).

The expression of p21 is regulated via p53; therefore, lack of p21 expression in some of the AML cell lines can also be attributed to a lack of p53. Mutations in the p53 gene have been reported for cell lines KG1, HL60, and U937 [2, 6]. Unsurprisingly, p53 cannot be detected by Western blot in these cells (data not shown), although p53-mutated U937 cells showed low but significant p53-independent expression of p21. Moreover, even after DAC exposure, the p53 protein level remained unchanged overall in the tested AML cells.

Although no change in p53 levels could be detected, HL60 cells with a known p53 mutation nevertheless exhibited low sensitivity to DAC, and p53-mutated KG1 cells responded in

a similar manner as p53-expressing OCI-AML2 cells to drug stimulation (data not shown). In conclusion, p53 expression does not correlate with the p21 induction or differences in sensitivity of the cells to DAC, suggesting that p53 is not a target for DAC.

Methylation of p21

The continued expression of p21 protein in p53 mutant AML cells prompted reverse transcriptase (RT)-PCR analysis of p21 transcription in p53-mutant KG1 and p53-expressing OCI-AML2 cells following DAC treatment (Fig. 2a). In both cell lines, p21 mRNA expression was increased after treatment with DAC (1 or 5 μM for 1 or 3 days).

p21 reexpression has previously been attributed to promoter methylation [18], prompting investigation of methylation of the 5′ region of p21. Using the methylation-sensitive restriction enzyme *Hpa*II and subsequent PCR, it was concluded that the p21 promoter was not methylated (Fig. 2b). The absence of promoter methylation was confirmed by bisulfite genomic sequencing (data not shown).

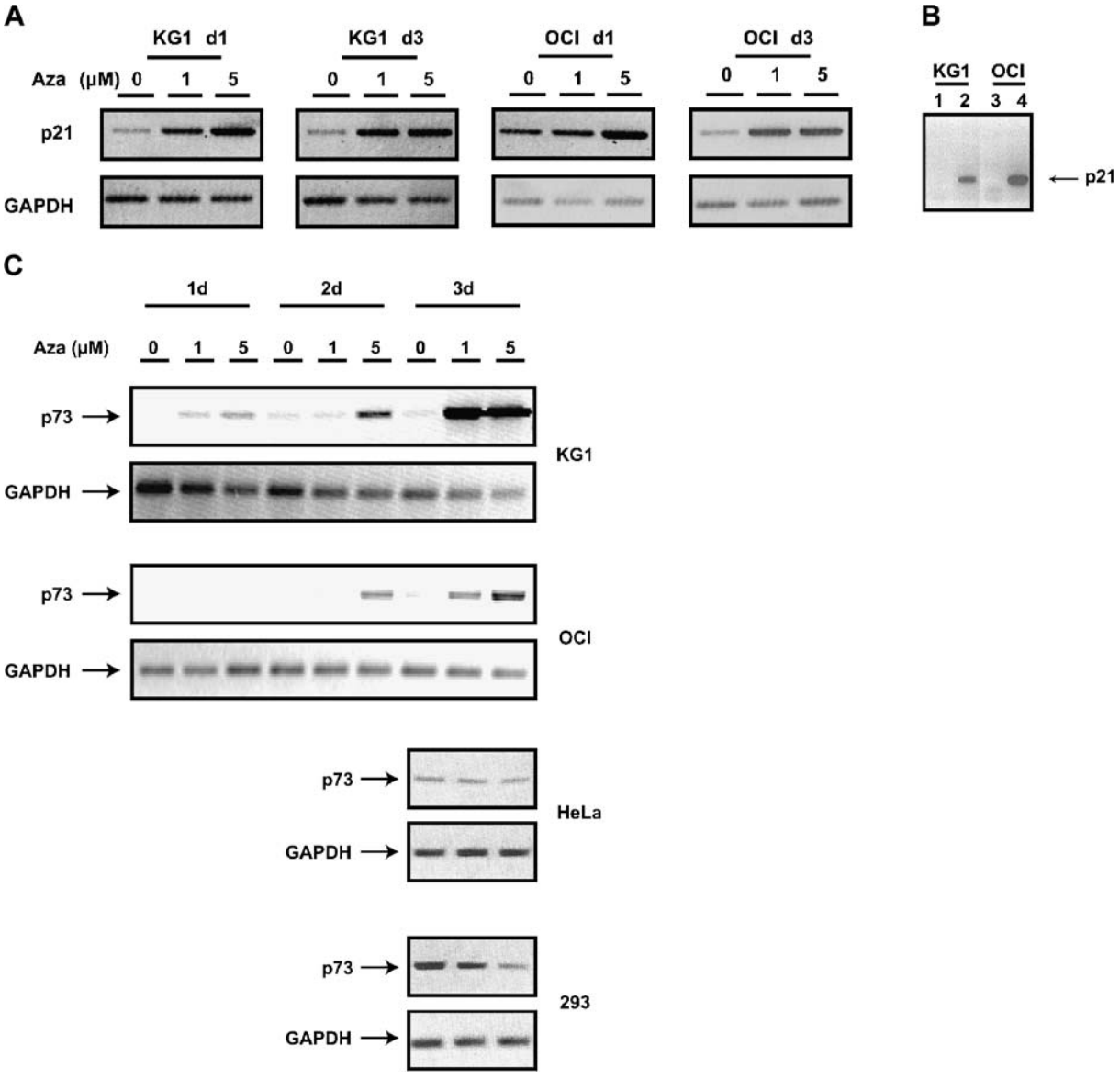

Fig. 2 Decitabine induces p21 mRNA upregulation without p21 promoter methylation. **a** RT-PCR analysis of KG-1 and OCI-AML2 cells treated as indicated for 1 (*d1*) or 3 days (*d3*) with decitabine. An increase in p21 mRNA compared to GAPDH as control gene is detectable. **b** Genomic DNA of untreated cells was digested with the methylation-sensitive restriction enzyme *Hpa*II (*lanes 1* and *3*) or was left undigested as control (*lanes 2* and *4*) followed by PCR amplification of the 5′ upstream region (−167 to −56) of CDKN1A (p21). PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining (**a** + **b**). **c** RT-PCR analysis of p73 mRNA induction following decitabine treatment standardized to GAPDH. p73 was unregulated in AML cell lines (KG1 and OCI-AML2) but not in epithelial cells (293 and HeLa) exposed to 0, 1, or 5 μM decitabine for 3 days as indicated. Reprinted with permission from Wiley and Sons [19]

These results suggest that DAC induces p21 expression independently of p21 gene methylation status and p53 deficiency.

Expression and methylation status of p73

p73 acts as a tumor suppressor similar to p53 [15]. p73-dependent induction of p21 has been reported previously [15]. The lack of p21 promoter methylation led to the hypothesis that p73 might be a target for DAC-mediated demethylation in AML. Decitabine caused time- and dose-dependent reexpression of p73 mRNA in KG1 and OCI-AML2 cells, whereas p73 mRNA induction was not observed in the DAC-resistant epithelial cell lines 293 and HeLa (Fig. 2c).

Methylation-specific PCR, using genomic DNA of cells exposed to 0, 1 or 5 μM DAC for 3 days, showed strong amplification in reactions specific for unmethylated forms, indicating that DAC caused demethylation of the promoter region of p73 in OCI-AML2 and U973 cells (Fig. 3a). MSP, using genomic DNA from untreated KG1 cells, showed a positive signal in reactions specific for both unmethylated and methylated forms, indicating incomplete methylation. However, after treatment of KG1 cells with DAC, the methylated genomic fraction decreased to a level similar to that in OCI-AML2 cells (Fig. 3a). In conclusion, in the AML cell lines OCI-AML2 and KG1, there is a clear correlation between p73 hypermethylation and promoter demethylation by DAC, which results in induction of p73 mRNA expression, ultimately causing apoptosis.

To assess the relationship between DNA demethylation and induction of apoptosis, the effect of DAC on p73 expression in epithelial cell lines that are resistant to DAC-induced apoptosis was analyzed. These epithelial cells (293 and HeLa) were found to be completely unmethylated for p73 (Fig. 3a). Treatment with DAC did not alter this methylation status or the expression level of p73 mRNA, suggesting that DAC was not able to induce p73 transcription in epithelial cells (Fig. 2c).

In all analyzed AML cell lines, DAC-mediated cytotoxicity correlated with its ability to revert p73 methylation and to reexpress p73 transcript. To explore whether these p73 methylation patterns were also seen in vivo, 86 peripheral blood and bone marrow samples taken from newly diagnosed, untreated AML patients were analyzed by MSP. The methylated epigenotype for p73 was found in 37.2% of the patient samples, while none of six PBMC samples from healthy donor controls were methylated at the p73 5′ region as described earlier (Fig. 3b) [13].

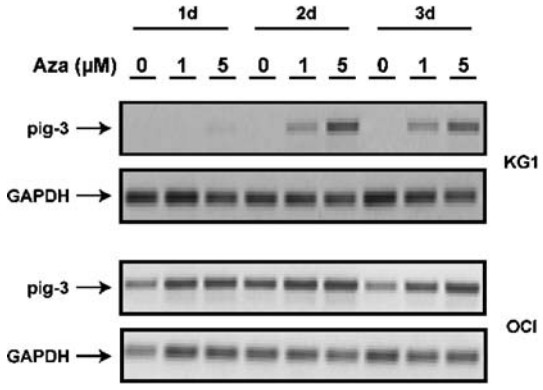

Fig. 4 Decitabine induces pig3 mRNA upregulation. RT-PCR analysis of KG-1 and OCI-AML2 cells treated as indicated for 1 (*d1*) or 3 days (*d3*) with decitabine. An increase in pig3 mRNA compared to GAPDH as control gene is detectable

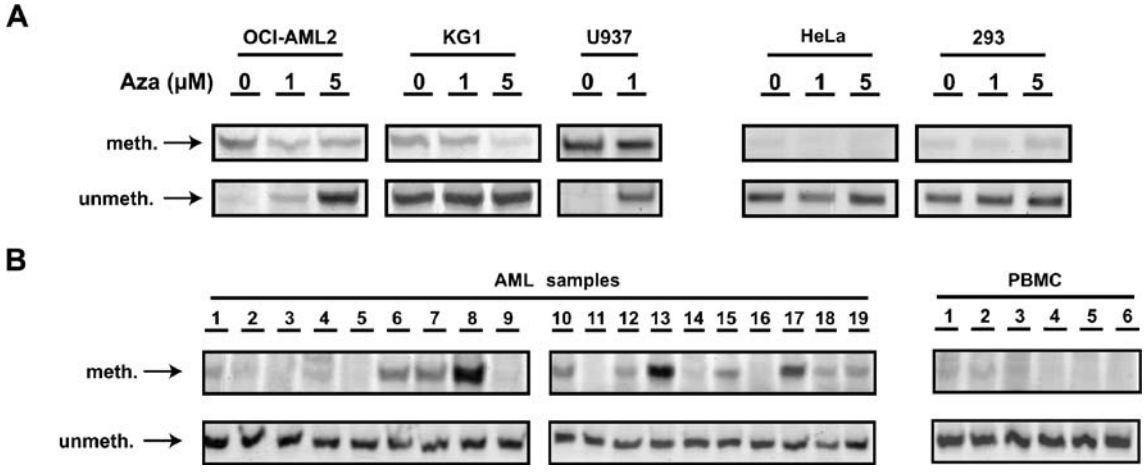

Fig. 3 Methylation of the p73 promoter as measured by MSP PCR using bisulfite-treated genomic DNA. The presence of a PCR product in the *upper lane* (*meth.*) or the *lower lane* (*unmeth.*) represent the presence of methylated or unmethylated 5′ region of p73, respectively. **a** Progressive promoter demethylation in AML but not in epithelial cell lines after a 3-day treatment with a single dose of decitabine. **b** Representative MSP results of AML primary samples and normal PBMCs of healthy donors did not show any methylation. In contrast, some of the AML patients displayed strong methylation of p73. Reprinted with permission from Wiley and Sons [19]

Expression of proapoptotic pig3

Reactivation of p73 by DAC resulted in apoptosis in both KG-1 and OCI-AML2 cells, while caspase-3 activity was absent in OCI-AML2 cells. To find mediators of DAC-induced apoptosis downstream of p73, we analyzed the expression of proapoptotic pig3 in these cells. Pig3 has been described as a transcriptional target of p73 involved in generating radical oxygene species (ROS) and activating apoptosis [21]. We found induction of pig3 mRNA in KG-1 and OCI-AML2 cells after application of a single dose of 1 or 5 μM DAC (Fig. 4), proposing pig3 as a candidate target gene of reactivated p73.

Discussion

Decitabine is a demethylating agent with the ability to reinduce expression of leukemia-related genes [11]. Therapeutic use of low-dose DAC seems very encouraging in older patients with MDS and AML, improving survival and reducing myeloid blast counts [10, 12, 22]. In contrast, the clinical efficacy of DAC in solid tumors appears to be limited [1].

This study was intended to correlate the inhibitory effect of DAC on DNA methylation with its ability to induce apoptosis by investigating DAC-inducible, methylation-regulated target genes that may be involved in mediating the cytotoxic effects of the drug in AML cells.

The presented results suggest that DAC induces apoptosis by activating specific caspases in AML but not in epithelial cells, hence displaying cell-selective cytotoxicity. In AML cell lines, DAC-induced apoptosis seems to be reliant on activation of the effector caspase-3 since no caspase-6 or caspase-8 processing was apparent. Decitabine-induced cell death in AML cells appears to occur via recruitment of the mitochondrial apoptosis pathway, a finding that is also supported by data regarding cytochrome *c* release and loss of mitochondrial membrane potential following drug treatment. In our panel of AML cell lines, extensive mitochondrial activation and subsequent apoptosis correlated with high sensitivity to DAC, suggesting that the damage to the mitochondrial integrity is an integral part of DAC-induced apoptosis in AML cells.

Interestingly, we found induction of apoptosis in one of the AML cell lines (OCI-AML2) to occur without activation of caspase-3, indicating that DAC can also recruit caspase-independent apoptosis pathways.

Some DAC-treated AML cell lines arrested in the G1 phase, and this correlated with increased expression of the cell cycle inhibitor p21. While previous publications have suggested that p21 silencing may be caused by hypermethylation of the p21 promoter [18], our data strongly suggest that in AML cells, the induction of p21 mRNA expression by DAC is independent of p21 methylation status. This result is in line with previous findings that DNA demethylation is not required for p21 induction [14]. In addition, reduction of the DNMTase level by means of antisense oligonucleotides has been shown to increase p21 protein independently of the methylation status of p21/CDKN1A in cancer cell lines [7, 16].

Although p21 is known to be a major transcriptional target of p53 [5], expression of p21 did not correlate with expression of the transcriptional regulator p53. The lack of p21 expression could be explained by a loss of p53 expression due to gene mutation. Indeed, expression of p53 was not detected in those cell lines at baseline or following DAC stimulation. Nevertheless, those cell lines were sensitive to DAC, suggesting that the response to DAC in AML cell lines is independent of p53.

The tumor suppressors p73 and p63 have been identified as members of the p53 family of transcription factors with high sequence homology to p53 [23]. p73 shares many functions with p53, such as cell cycle control and regulation of apoptosis, and is therefore an evident candidate to regulate DAC-induced p21 expression and subsequent apoptosis. In contrast to p53, p73 functional mutations in myeloid leukemias are rare, although monoallelic expression has been described [20].

In the studied AML cell lines, a strong correlation between p73 hypermethylation and expression levels of p73 was found. Decitabine caused promoter demethylation, resulting in induction of p73 mRNA reexpression and subsequent apoptosis in AML but not epithelial cell lines. Thus, p73 is a candidate target gene for DAC-mediated cytotoxicity in AML cells.

Recently, p73 function has been linked to chemosensitivity, e.g., toward etoposide and doxorubicin [9]. The blocking of p73 by small interfering RNA and other strategies led to chemo resistance irrespective of the p53 mutation status in these cells [9]. A recent report demonstrated that p73 triggers apoptosis via p53-upregulated modulator of apoptosis (PUMA)-mediated activation of the mitochondrial apoptosis pathway [15]. We found a correlation between DAC-mediated apoptosis and its ability to induce p21 mRNA as a consequence of p73 transactivation. Today, it remains unclear whether induction of p21 as a downstream target gene of p73 alone is sufficient to explain the cytotoxicity of DAC in AML cells. Further studies will be necessary to dissect the role of other p73 target genes such as pig3 or PUMA in DAC-induced apoptosis in AML cells.

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