Induction of the Proapoptotic Tumor Suppressor Gene Cell Adhesion Molecule 1 by Chemotherapeutic Agents Is Repressed in Therapy Resistant Acute Myeloid Leukemia

Muriel C. Fisser, Anna Rommer, Katarina Steinleitner, Gerwin Heller, Friederike Herbst, Meike Wiese, Hanno Glimm, Heinz Sill, and Rotraud Wieser

1Department of Medicine I, Medical University of Vienna, Vienna, Austria
2Comprehensive Cancer Center of the Medical University of Vienna, Vienna, Austria
3Department of Translational Oncology, National Center for Tumor Diseases (NCT) and German Cancer Research Center (DKFZ), Heidelberg, Germany
4German Consortium for Translational Cancer Research (DKTK), Heidelberg, Germany
5Division of Hematology, Medical University of Graz, Graz, Austria

Even though a large proportion of patients with acute myeloid leukemia (AML) achieve a complete remission upon initial therapy, the majority of them eventually relapse with resistant disease. Overexpression of the gene coding for the transcription factor Ecotropic Virus Integration site 1 (EVI1) is associated with rapid disease recurrence and shortened survival. We therefore sought to identify EVI1 target genes that may play a role in chemotherapy resistance using a previously established in vitro model system for EVI1 positive myeloid malignancies. Gene expression microarray analyses uncovered the Cell Adhesion Molecule 1 (CADM1) gene as a candidate whose deregulation by EVI1 may contribute to drug refractoriness. CADM1 is an apoptosis inducing tumor suppressor gene that is inactivated by methylation in a variety of tumor types. In the present study we provide evidence that it may play a role in chemotherapy induced cell death in AML: CADM1 was induced by drugs used in the treatment of AML in a human myeloid cell line and in primary diagnostic AML samples, and its experimental expression in a cell line model increased the proportion of apoptotic cells. CADM1 up-regulation was abolished by ectopic expression of EVI1, and EVI1 expression correlated with increased CADM1 promoter methylation both in a cell line model and in primary AML cells. Finally, CADM1 induction was repressed in primary samples from AML patients at relapse. In summary, these data suggest that failure to up-regulate CADM1 in response to chemotherapeutic drugs may contribute to therapy resistance in AML.

Key words: acute myeloid leukemia; chemotherapy resistance; relapse; EVI1; CADM1
and relapse of AML, which are regarded chemotherapy responsive and resistant stages of the disease, respectively.

The Cell Adhesion Molecule 1 (CADM1) gene (also known as TSLC1, IGSF4, or syncAM), which codes for a transmembrane glycoprotein, is downregulated in a variety of solid tumors - most often through methylation of the CpG island (CGI) at its 5’ end, but also due to gene deletion or other, still unknown mechanisms [3–7]. In some cancer types, methylation and/or downregulation of CADM1 was shown to be associated with advanced disease and/or a dismal outcome [4,5,7,8]. Mice with a homozygous disruption of the Cadm1 locus displayed increased susceptibility to both spontaneous and experimentally induced tumor formation [9]. Conversely, ectopic expression of CADM1 suppressed tumor growth in xenograft models, which was accompanied by elevated levels of apoptosis in some studies [3,4,10]. In vitro, increased rates of apoptotic cell death were also observed upon ectopic expression of CADM1 [4,6,10]. Heat shock and serum withdrawal upregulated the CADM1 gene, but this induction was prevented by promoter methylation [6].

In AML, CADM1 promoter methylation, present in 33% of cases, correlated with adverse cytogenetics, and was associated with shortened overall survival in an epigenetic background defined by an unmethylated ESR1 gene [8]. Childhood AML patients with the prognostically favourable translocation t(9;11)(p22; q23) expressed higher levels of CADM1 than patients with other MLL rearrangements [11], and an inverse correlation between EVII and CADM1 expression was present in AML with MLL rearrangements [12].

We recently reported that experimental expression of EVII increased the resistance of human myeloid cells towards cytostatic drugs [13]. Gene expression microarray analyses using U937_EVII and U937_vec cells, which had been infected with a retroviral EVII expression vector or empty control vector, respectively, revealed several candidate genes that may contribute to EVII induced chemotherapy resistance [13]. Among them, the CADM1 gene was induced by etoposide in cells lacking, but not in cells expressing, EVII. In the present study, we investigated the induction of CADM1 by chemotherapeutic drugs, its repression by EVII, and its potential contribution to apoptosis in human myeloid cells in more detail.

**MATERIALS AND METHODS**

U937_vec and U937_EVII cells were cultured as described previously [13]. Incubations with 400 nM etoposide, 30 nM daunorubicin, or 40 nM cytosine arabinoside (araC) were carried out for 48 h. Demethylating treatment was performed by addition of 11 μM freshly dissolved 5-aza-2-deoxycytidine (azaC, Sigma-Aldrich, Seelze, Germany) to U937_vec and U937_EVII cells on each of four consecutive days. On the fourth day, 440 nM trichostatin A (TSA, Sigma) was also added, and cells were harvested another 24 h later. Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA) and reverse transcribed using random hexamer primers (Life Technologies) and M-MLV reverse transcriptase (Life Technologies). mRNA levels of CADM1 and of the housekeeping gene Beta-2-Microglobulin (B2M) were measured in triplicate using a Step One Plus Real Time PCR system (Applied Biosystems, Life Technologies) and TaqMan Gene Expression Assays (CADM1: Hs00942509_m1*, B2M: 4333766F; Applied Biosystems).

For bisulfite conversion of genomic DNA and subsequent methylation sensitive high resolution melting curve analysis (MS-HRM) analysis, the Epitope Bisulfite and HRM PCR kits (Qiagen, Hilden, Germany) were employed. Primers CADM1-CpG-F (5’-GAGGATTTTTTTAAGGGAGAT-3’) and CADM1-CpG-R (5’-TCAAAAAAAATAATTTTCC-C3’) amplify a fragment from –297 to –148 relative to the CADM1 transcriptional start site. All measurements were carried out in duplicate, and a standard curve containing fully methylated and fully unmethylated DNA in varying proportions (100, 75, 50, 25, 10, and 0% of fully methylated DNA) was included in each experiment.

The human CADM1 cDNA [14] was cloned into the retroviral vector pMIA-II_JRES-Ametrine to yield pMIA-II_CADM1, which was infected into U937_vec and U937_EVII cells using standard procedures. Expression of ectopic CADM1 was confirmed by immunoblot analysis using rabbit anti-syncAM antibody ab3910 (Abcam, Cambridge, UK), and mouse mAb anti-β-tubulin clone TUB 2.1 (Sigma-Aldrich, Seelze, Germany) as a loading control. Only freshly infected, Ametrine positive cells were subjected to staining with annexinV-APC and 7-AAD (both from BD Biosciences, San Jose, CA). For all assays employing cell lines, at least three biological replicate experiments were performed, and means +/- standard errors of the means (SEMs) are shown. Statistical significance was calculated using Student’s t-test (2-tailed).

Experiments with primary human samples were approved by the ethics committee of the Medical University of Vienna. For MS-HRM based methylation analyses, archival samples were used. Twelve patients (median age, 53 years [16–83]; 7 female, 5 male) did not overexpress EVII based on previous qRT-PCR results [1], and 10 patients (median age, 56 years [20–82]; 7 female, 3 male; 6 with inv(3)(q21q26), 4 with t(3;3)(q21q26)) were inferred to do so based on the presence of 3q26 rearrangements [2]. For samples used to investigate CADM1 induction at diagnosis and relapse (Table 1), informed consent was obtained prior to their collection. Leukemic blasts were enriched by centrifugation through a Ficoll gradient and vitally frozen. For culture in the presence or absence of araC, thawed cells were first incubated with DNasel to avoid clumping due to dying cells, and

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then transferred for 48 h to RPMI supplemented with 10% FCS (Life Technologies) and 100 ng/ml each of IL-3, G-CSF, and SCF (Peprotech, Hamburg, Germany), as well as 4 μM araC where indicated. Cellular viability after araC treatment was between 24 and 54%, as determined by the Cell Titer Glo assay (Promega, Madison, WI).

RESULTS AND DISCUSSION

To confirm and extend the microarray results, RNA from U937_vec and U937_EVI1 cells cultured in the absence or presence of etoposide, daunorubicin, or araC for 48 h was subjected to quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). In agreement with the microarray data, CADM1 was up-regulated in response to etoposide in U937_vec cells, while in U937_EVI1 cells basal CADM1 mRNA levels were repressed, and no induction by etoposide was observed (Figure 1A). Similar results were obtained upon treatment with daunorubicin or araC (Figure 1A). Because EVI1 was recently shown to interact with DNA methyl transferases, leading to methylation of CGIs of some of its target genes [15], and because the CADM1 5' region harbours a CGI

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which is methylated in a number of tumor types, including a subset of AML [8,11], we asked whether repression of CADM1 in U937_EVI1 cells was associated with increased promoter methylation. Genomic DNA isolated from U937_vec and U937_EVI1 cells was treated with sodium bisulfite and subjected to MS-HRM analysis. Methylation levels of the CADM1 CGI were already relatively high in U937_vec cells, yet significantly higher levels were observed in U937_EVI1 cells (Figure 1B). Next, qRT-PCR was performed on RNA extracted from U937_vec and U937_EVI1 cells after treatment with or without the DNA methyltransferase inhibitor azaC. In concordance with the results of the MS-HRM analysis, azaC caused an induction of CADM1 in both cell lines, but the effect was more pronounced and reached statistical significance only in U937_EVI1 cells (Figure 1C). To investigate whether EVII expression was associated with CADM1 methylation also in primary AML samples, MS-HRM analysis was performed on DNA from 12 patients not expressing EVII based on previous qRT-PCR results [1], and from 10 patients with 3q26 rearrangements. These experiments showed that 3q26 rearrangements, and by inference, EVII overexpression [2], were associated with significantly increased methylation of the CADM1 CGI (Figure 1D).

CADM1 has been reported to promote apoptotic cell death in several different cell types [4,6,10]. To test whether this was also true for human myeloid cells, U937_vec and U937_EVI1 cells were infected with pMIA-II_CADM1 or with empty vector as a control, and sorted for Ametrine positivity. Expression of the exogenous CADM1 gene was confirmed by immunoblot analysis (Figure 2A). Flow cytometric analysis of annexin V/7-AAD stained cells showed that overexpression of CADM1 caused significantly increased rates of apoptosis in both U937_EVI1 and U937_vec cells (Figure 2B).

Finally, to determine whether the ability to upregulate CADM1 was of more general relevance in the response of AML cells to chemotherapeutic agents, primary AML samples were cultured in the presence or absence of aracC for two days and subjected to qRT-PCR. CADM1 was induced by araC in all three investigated diagnostic samples (Figure 2C), but...
interestingly, samples from the same patients at the time of relapse showed greatly decreased basal CADM1 levels in two out of three cases, and induction by araC was almost entirely abolished in all three cases (Figure 2C). However, no substantial differences in CADM1 promoter methylation were found between diagnosis and relapse in any of the three patients (not shown).

In summary, our data show for the first time that CADM1, which acted in a pro-apoptotic manner in human myeloid cells, was induced by agents used in AML chemotherapy both in a human myeloid cell line and in primary AML samples, and that this induction was lost in EVI1 overexpressing cells as well as in relapsed AML. While EVI1 mediated CADM1 repression was associated with CGI methylation, other, yet to be identified mechanisms appeared to be responsible for repression at relapse. These findings suggest that further investigations of the role of CADM1 in chemotherapy induced apoptosis, and of its downregulation in drug resistant disease, may reveal novel targets for molecularly directed therapies in AML.

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