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Activation of HLA-G expression by 5-aza-2′- deoxycytidine in malignant hematopoetic cells isolated from leukemia patients

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Human leukocyte antigen – G (HLA-G) is a non-classical HLA class I antigen with restricted distribution in normal tissues. Ectopic HLA-G expression observed at some pathological circumstances as malignant transformation might be triggered by epigenetic modifications such as DNA demethylation. Recently it was demonstrated that DNA methyltransferase inhibitor 5-aza-2′- deoxycytidine (AdC) induces/enhances HLA-G transcription in many leukemia cell lines of different origin. Here we investigated the effect of AdC on HLA-G expression in malignant hematopoetic cells isolated from patients with acute myeloid leukemia (AML) and chronic lymphocytic leukemia (B-CLL). We detected HLA-G expression in untreated cells from some patients. Nevertheless treatment with 5-aza-2′- deoxycytidine enhanced HLA-G transcription and concomitantly HLA-G protein synthesis in some leukemia cells.

Key words: HLA-G, DNA demethylation, 5-aza-2′- deoxycytidine, AML, B-CLL

HLA-G is a non-classical HLA class I antigen primarily expressed in the extravillous cytotrophoblast [1, 2]. Ectopic HLA-G expression was also reported in a number of pathological tissues as tumors or virus infected cells [3–6]. The regulation of *HLA-G* gene expression is different to that of classical *HLA class I* genes because almost all known regulatory sequences for classical *HLA class I* genes are disrupted [7–9]. Recent studies demonstrated that regulation of *HLA-G* gene activity also involves epigenetic mechanisms such as DNA demethylation or histone acetylation [10–14]. Furthermore, some stress conditions as hypoxia or heat shock may up-regulate HLA-G transcriptions [15–17].

HLA-G antigen exerts multiple immunoregulatory functions such as inhibition of natural killer cells, cytotoxic T lymphocytes and induction of T-cell apoptosis. The presence of HLA-G on cytotrophoblasts contributes to maternal-fetal tolerance. Similarly the ectopic expression of HLA-G antigens on tumor cells may help them to escape from immune response [18–20]. 5-aza-2'-doexycytidine (AdC) is a DNA hypomethylating agent that inhibits DNA methyltransferase [21, 22]. Due to DNA demethylation by AdC the synthesis of immunosuppressive HLA-G molecules can be induced. In our previous study we demonstrated that AdC treatment increased HLA-G mRNA expression in many human leukemia cell lines, and that the up-regulation of gene transcription was often accompanied with increased level of HLA-G protein [14]. Although 5-aza-cytidine (Vidaza) and 5-aza-2′- deoxycytidine (Decitabine) were recently approved by FDA for leukemia and myelodysplastic syndromes (MDS) therapy [23–25], the effect of AdC on HLA-G expression in leukemia cells from patients has not been examined until now. Therefore in the present work we studied the efficacy of AdC on HLA-G gene transcription in malignant hematopoetic cells freshly isolated from patients with AML or B-CLL. We found that treatment with 5-aza-2′- deoxycytidine enhanced HLA-G transcription and also HLA-G protein synthesis in some leukemia specimens.

Materials and methods

Cells and cell treatment. Patient samples were taken for diagnostic purposes and surplus material was used in this experimental study after informed content of patient. Patients diagnosed *de novo* with AML or B-CLL are characterized in Table 1. Diagnosis was performed by morphological and cytochemical criteria and by immunophenotyping. Mononuclear

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cells were isolated from peripheral blood of newly diagnosed and untreated patients by standard Ficoll-Hypaque density gradient centrifugation. Freshly isolated cells were resuspended in RPMI 1640 medium supplemented with 2mM L-glutamine, 200 μg/ml gentamicin, 0.125 μg/ml amphotericin B and 10% heat-inactivated fetal bovine serum. Demethylating treatment of cells was carried out with 100 μM 5-aza-2´- deoxycytidine (Sigma) for 3 days.

Human choriocarcinoma cell lines JEG3 and JAR (ATCC, Rockville, MD) were used as HLA-G positive and negative controls, respectively.

Semiquantitative RT-PCR analysis. RT-PCR analysis was performed according the procedure described previously [14]. Briefly, total RNA was extracted with Trizol reagent (Life Technologies, Gaithersburg, MD, USA). Reverse transcription (RT) was carried out with Taq Man Reverse Transcription Reagents (Applied Biosystems) at 42 °C for 60 min, and stopped by heating at 95 °C for 5 min. PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems) using pan HLA-G primers (G.257-F, G.1004-R) as described in Table 2. As an internal control the ß-actin gene amplification was carried out for each sample with primers BGU-F and BGL-R (Table 2). The PCR started by incubation at 95 °C for 15 min, followed by PCR cycles of 1 min at 94 °C, 1.5 min at 62 °C, 2 min at 72 °C, with a final extension at 72 °C for 7 min. The number of PCR cycles was determined experimentally: 37 cycles for HLA-G and 25 cycles for ß-actin. PCR products were visualized by UV light after electrophoresis in 2% agarose gels stained with ethidium bromide.

Real-time RT-PCR expression analysis. Real-time PCR reactions were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). A singleplex reaction mix was prepared containing TaqMan Universal PCR Master Mix, 200 nM of specific primers, 150 nM of specific TagMan probe. cDNA from 25 ng total RNA was amplified in a 25 μl reaction mixture. The thermal cycling conditions included an initial activation of DNA polymerase 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. TaqMan probes containing 6-carboxyfluorescein at the 5'end (FAM reporter) and 6-carboxytetramethylrhodamine at the 3'end (TAMRA quencher) that hybridize to a sequence located between PCR primers, were used. HLA-G specific primers and probe were selected to amplify all alternative forms of HLA-G transcripts (Table 2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers and probe were used as endogenous control (Table 2). Data were analyzed using the $2^{-\Delta\Delta CT}$ method and reported as the fold change in HLA-G gene expression in experimental cell lines normalized to the endogenous control gene (GAPDH) and relative to the control (JEG3 cells assigned a value of 1). Cycle threshold (CT) values were determined by automated threshold analysis with ABI Prism software. CT values of endogenous control GAPDH were not influenced under the used experimental conditions.

Western blot analysis. Western blot analysis was accomplished by standard method as previously described [14]. Briefly, cell pellets were lysed at 4 °C in TENN buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl,1 mM EDTA, and 1% Nonidet P-40) supplemented with protease inhibitors. Cell extracts were boiled in SDS-PAGE reducing sample buffer and applied on 10% SDS-polyacrylamide gel. Following electrophoresis, the proteins were electro-blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, USA). Then the membrane was blocked with 5% non-fat powder milk in PBS and incubated with mAb 4H84 (a generous gift from Dr. McMaster, San

Table 1. Characteristics of patients

patient	gender/age	leukemia
PJ	M / 63	B-CLL
RJ	M / 57	B-CLL
HF	M / 48	B-CLL
ML	M / 69	B-CLL
HJ	M / 51	B-CLL
RL	M / 48	AML-M2
KP	M / 50	AML-M3
MT	M / 20	AML-M5
BM	F / 48	AML-M2
TF	M / 78	AML-M0
GZ	F / 58	AML-M3
KM	M / 21	AML-M3
PS	F / 53	AML-M4
TK	F / 47	AML-M4/M5

Table 2. Primers and probes for RT-PCR analysis

Primers for semiquantitative RT-PCR	
Name	Sequence
G.257-F	5'-GGAAGAGGAGACACGGAACA -3'
G.1004-R	5'-CCTTTTCAATCTGAGCTCTTCTTT -3'
BGU-F	5'-ATGTTTGAGACCTTCAAC -3'
BGL-R	5'-CACGTCACACTTCATGATGG -3'
Primers and probes for TaqMan real-time RT-PCR	
Name	Sequence
GAPDH-F	5'-CATGGGTGTGAACCATGAGAA-3'
GAPDH-R	5'-GGTCATGAGTCCTTCCACGAT-3'
GAPDH probe	5'-AACAGCCTCAAGATCATCAGCAATGCCT-3'
HLA-G-F	5'-CTGGTTGTCCTTGCAGCTGTAG-3'
HLA-G-R	5'-CCTTTTCAATCTGAGCTCTTCTTTCT-3'
HLA-G probe	5'-CACTGGAGCTGCGGTCGCTGCT-3'

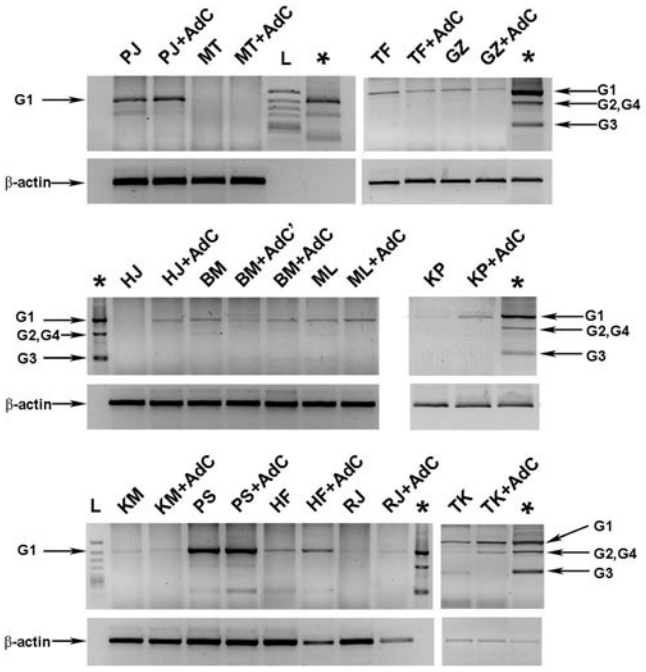

Figure 1. Semi-quantitative RT-PCR analysis of HLA-G transcripts (G1-G4) following AdC treatment of mononuclear cells isolated from leukemia patients.
Mononuclear cells isolated from patients with B-CLL or AML were untreated or treated with 100 μM 5-aza-2´-deoxycytidine for days 3 (+AdC) or 2 (+AdC'). L: DNA ladder. Asterisks: untreated cells JEG3 were used as a HLA-G positive control.

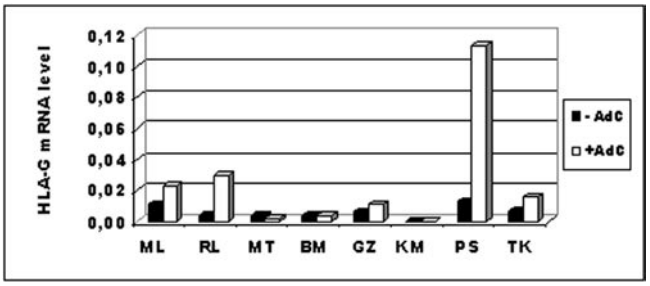

Figure 2. Real-time RT-PCR analysis of HLA-G mRNA expression following AdC treatment of mononuclear cells isolated from leukemia patients.
Mononuclear cells isolated from leukemia patients were untreated (-AdC) or treated with 100 μM 5-aza-2´-deoxycytidine for 3 days (+AdC). HLA-G mRNA levels in cell lines were compared to JEG-3 cells (assigned a value of 1).

Francisco, CA, USA), recognizing free heavy chain of HLA-G antigen. After extensive washing the membrane was incubated with peroxidase-conjugated rabbit anti-mouse IgG antibodies (Dako, Hamburg, Germany), subsequently washed and the immunoreactive bands were visualized with chemoluminescence reagent (ECL Detection System Santa Cruz Biotechnology, Inc., Santa Cruz,USA).

Results

Activation of HLA-G transcription in malignant hematopoetic cells following 5-aza-2´- deoxycytidine (AdC) treatment. Regulation of *HLA-G* gene activity could involve epigenetic mechanisms such as DNA demethylation. We have studied the effect of DNA methylation inhibitor 5-aza-2´- deoxycytidine (AdC) on HLA-G transcription in mononuclear cells isolated from 14 patients diagnosed with *de novo* B-ALL and AML (Table 1). The choriocarcinoma cell line JEG-3 was used as a HLA-G positive control. First the HLA-G transcription was analyzed by semi-quantitative RT-PCR which allows recognition of the alternative HLA-G transcripts. The enzyme AmpliTaq Gold DNA Polymerase was used to enhance PCR amplification yield and specificity of reaction. Under such conditions, the HLA-G1 transcript was detected in some untreated mononuclear cells isolated from patients with B-CLL (PJ, HF, ML) or AML (BM, TF, GZ, PS, TK) (Fig. 1). Particularly high level of HLA-G1 isoform was demonstrated in samples of patients PJ (B-CLL) or PS (AML). After AdC treatment mostly the up-regulation of HLA-G1 mRNA was observed with exceptions of PS and TK samples where also HLA-G3 or HLA-G2/G4 isoforms were found (Fig.1). Such up-regulation also resulted into detection of HLA-G transcripts in several originally negative cell samples (HJ, KP). Some cell samples (RJ, MT, KM) following AdC treatment did not express HLA-G transcripts at all or the level of transcripts was below the detection threshold. Real time-PCR confirmed significant increase in HLA-G transcription following AdC treatment for cells from patients ML, RL, GZ, PS and TK (Fig. 2). Altogether there was a good agreement between results obtained by semiquantitative RT-PCR (Fig. 1) and real time RT-PCR (Fig. 2).

Expression of HLA-G protein in malignant hematopoetic cells before and after AdC treatment. Next we studied HLA-G protein expression in hematopoetic cells isolated from the same group of patients with B-ALL or AML. The total HLA-G protein in cells was examined by western blot analysis using mAb 4H84. The choriocarcinoma cell line JAR treated with 100 μM AdC for 3 days served as a HLA-G positive control.

We have demonstrated that in leukemic cells with remarkable level of HLA-G mRNA, a significant amount of total HLA-G1 protein was also present (Fig. 3). In those hematopoetic cells, where AdC treatment considerably up-regulated *HLA-G* gene transcription, a higher amount of HLA-G protein was also detected, especially in samples PS (AML) and ML (B-CLL). Generally we can conclude that the total HLA-G protein expression in examined cells correlates well with the level of HLA-G transcripts (Table 2).

Expression of HLA-G transcripts in hematopoetic cells isolated from patient after allogenic stem cell transplantation. From previous experiments it is clear that mononuclear cells isolated from peripheral blood of PS patient with AML (60% blasts in peripheral blood and 83% blasts in bone marrow) expressed

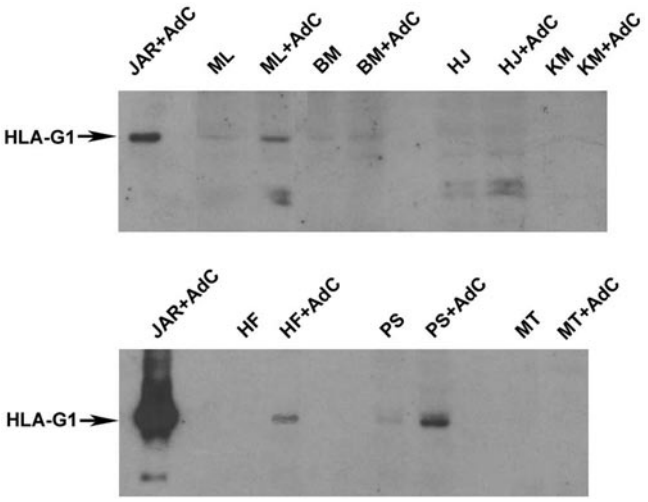

Figure 3. Western blot analysis of HLA-G protein following AdC treatment of mononuclear cells isolated from leukemia patients.
Total expression of HLA-G proteins in leukemia cell lines untreated or treated with 100 μM AdC for 3 days (+AdC) was analyzed by western blotting using mAb 4H84 (specific for all denatured HLA-G isoforms). Choriocarcinoma cell line JAR treated with AdC was used as a HLA-G positive control.

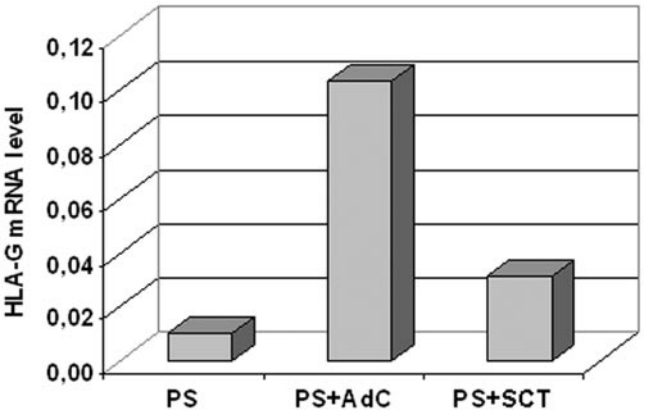

Figure 4. Real-time RT-PCR analysis of HLA-G mRNA expression in mononuclear cells isolated from PS patient before and after allogenic stem cell transplantation.
PS: cells from AML patient PS, PS+AdC: cells isolated from AML patient PS and treated with 5-aza-2′- deoxycytidine, PS+SCT: cells from patient PS after allogeneic hematopoetic stem cell transplantation. HLA-G mRNA levels were compared to JEG-3 cells (assigned a value of 1).

significant amounts of HLA-G mRNA as well as HLA-G1 protein and that the levels of both were up-regulated following AdC treatment. The patient PS was submitted to allogeneic hematopoetic stem cell transplantation from HLA-identical sibling. On day +120 of transplantation without graft versus host disease (GVHD), the peripheral blood mononuclear cells were isolated from patient PS and the level of HLA-G transcripts was estimated by quantitative real-time RT-PCR. As documented in Fig. 4, HLA-G mRNA level in mononuclear cells isolated after transplantation was higher than in malignant hematopoetic cells obtained from the same patient before treatment, but lower than in malignant cells following AdC treatment.

Discussion

HLA-G is a non-classical major histocompatibility class I antigen characterized by a limited expression restricted mainly to the extravillous trophoblasts of human placenta [1, 2]. HLA-G antigens exhibit immunosuppressive properties such as inhibition of natural killer (NK) cytolysis and cytotoxic T lymphocyte (CTL) responses. As HLA-G molecules is expressed in some tumor cells it may play an important role in their immune escape mechanisms [18, 19]. The presence of HLA-G mRNA but not HLA-G protein in malignant hematopoetic cells from patients with various hematopoetic malignances as acute myeloid leukemia(AML), acute lymphoblastic leukemia(ALL), chronic lymphocytic leukemia (CLL) or non-Hodgkin lymphomas (NHL), was first reported by Amiot et al. [26]. Later on the surface HLA-G protein was detected on malignant cells of NHL, CLL, AML and multiple myeloma (MM) [27–30]. In the last years an increased level of sHLA-G proteins in serum of patients with NHL, AML, CLL and MM was also observed [31, 34]. Several investigators believe that expression of HLA-G protein (soluble or anchored on the cell membrane) is associated with unfavorable outcome of some hematopoetic malignances [27, 28].

It was demonstrated that in regulation of *HLA-G* gene transcription also epigenetic mechanisms such as DNA methylation or histone acetylation are involved [10–15]. We demonstrated that treatment with demethylating agent AdC resulted in up-regulation of HLA-G transcription in 18 out of 20 examined leukemia cell lines and expression of HLA-G protein in 10 cell lines [14]. Therapy of human hematopoietic malignances with AdC may induce/enhance the synthesis of immunosuppressive HLA-G molecules and thus contribute to tumor escape from the host immune system. Here we expanded our study of AdC effect on *HLA-G* gene transcription in leukemia cells isolated from patients with AML or B-CLL. First we examined the presence of HLA-G transcripts in AdC un-treated leukemia samples. HLA-G transcripts using RT-PCR were demonstrated in 3 out of 5 patients with B-CLL and in 5 out of 8 patients with AML. AdC treatment increased the level of HLA-G mRNA that was shown clearly by quantitative real time RT-PCR. The translation activity of HLA-G was also studied by western blot analysis. It was confirmed that patient's leukemia cells with high amount of HLA-G mRNAs also expressed HLA-G1 protein. The increase of HLA-G1 protein synthesis following AdC treatment correlates with up-regulation of *HLA-G* gene transcription (Table 3). These experiments clearly show that AdC treatment of human leukemia as AML or

Table 3. Summary of HLA-G mRNA and protein expression in leukemia cells following 5-aza-2´-deoxycytidine treatment

patient	semiquant.RT-PCR		real time RT- PCR		WB	
	-AdC	+AdC	-AdC	+AdC	-AdC	+AdC
B-CLL:						
PJ	++	++	NT	NT	NT	NT
RJ	-	-	NT	NT	NT	NT
HF	+	+	NT	NT	-	+
ML	+	+	0,011	0,023	+-	+
HJ	-	+	NT	NT	-	+-
AML:						
RL	NT	NT	0,004	0,031	NT	NT
KP	-	+	NT	NT	NT	NT
MT	-	-	0,004	0,002	-	-
BM	+	+	0,004	0,004	-	+-
TF	+	+	NT	NT	NT	NT
GZ	+	+	0,006	0,011	NT	NT
KM	-	-	0	0	-	-
PS	++	++	0,013	0,114	+-	++
TK	+	+	0,007	0,016	NT	NT

Cells were isolated from leukemia patients and treated with 100 μM AdC for 3 days.
Expression of HLA-G mRNA was analyzed by semiquantitative RT-PCR and real time RT-PCR. HLA-G protein expression was investigated by western blot analysis (WB).
The intensity of bands was scored as negative (-), weakly (+-), moderate (+), or markedly positive (++). NT: not tested.

B-CLL may induce HLA-G protein synthesis in some patients. Such up-regulation of HLA-G antigen might allow leukemia cells to escape from recognition and destruction of leukemia cells by cytotoxic T cells or NK cells.

Furthermore we have investigated the levels of HLA-G transcripts in mononuclear cells isolated from patient PS with AML before and after allogeneic peripheral blood stem cell transplantation. Real time RT-PCR analysis showed significantly higher level of HLA-G mRNAs in mononuclear leukocytes isolated from PS patient in the fourth month post transplantation than in leukemia cells obtained from the same patient before grafting. Because only one graft recipient was investigated in this work, we are unable to make any conclusion so far. However the expression of sHLA-G antigens in peripheral blood of leukemia patients after hematopoetic stem cell transplantation was recently examined [35]. The authors observed an increased plasma level of soluble HLA-G between the first and third month post allogeneic tranplantation in patients without acute graft versus host disease (GVHD). The cells responsible for production of these soluble HLA-G molecules following stem cell transplantation are unknown. It seems that blood mononuclears could be responsible for up-regulated production of sHLA-G and its subsequent release that in these cells was observed the up-regulation of HLA-G mRNA after renal transplantation [36]. The high levels of sHLA-G antigens were also detected in the plasma of patients with better acceptance of solid transplantants as heart, liver, and kidney-liver [37]. Expression of HLA-G antigen was also confirmed in allograft biopsies [38]. It was established that a higher ectopic HLA-G expression in grafts and/or in serum from organ transplant patients correlates with better graft acceptance [39]. The mechanism by which HLA-G is induced following transplantation treatment remains to be explained.

It follows from our results that DNA methylation is a widespread and important control mechanism of HLA-G gene expression. Nevertheless individual leukemia cell lines and likewise leukemia cells isolated from patients show vastly different response to the demethylation by AdC. This observation indicates that response to AdC treatment of human leukemia patients will be apparently also very different. Therefore patients should be monitored for HLA-G expression in order to follow risk of AdC therapy.

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