Modulation of HLA-G expression

K. POLAKOVA[1], E. BANDZUCHOVA[2], J. TIRPAKOVA[2], D. KUBA[2], G. RUSS[3]

[1]Cancer Research Institute, e-mail: exonpola@savba.sk, Slovak Academy of Sciences, 833 91 Bratislava, Slovak Republic; [2]Slovak Medical University, Bratislava, and [3]Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic

Received April 15, 2007

Recent studies demonstrated that HLA-G transcription is in some cells silenced by epigenetic mechanisms as DNA methylation and histone modification. Accordingly *HLA-G* gene transcriptions can be activated in such cells by demethylating agent or by inhibitors of histone deacetylation. In addition to epigenetic alterations *HLA-G* gene transcription can be activated by stress. In the present study these aspects of HLA-G expression are re-examined and a new inhibitor of histone deacetylation (valproic acid) and hypoxia mimetic chemical ($CoCl_2$) are included. The highest activation of HLA-G transcription was achieved by treatment of choriocarcinoma JAR and lymphoblastoid RAJI cell lines with demethylating agent 5-aza-2´-deoxycytidine. Treatment of JAR and RAJI cells with histone deacetylase inhibitors (sodium butyrate and valproic acid) also enhanced HLA-G transcription. Nevertheless this increase in HLA-G expression was low as compared with activation by 5-aza-2´- deoxycytidine. The hypoxia mimetic agents (desferrioxamine or $CoCl_2$) had no detectable effect on *HLA-G* gene transcription in examined cells. Relatively high increase of HLA-G transcription was detected in JAR and RAJI cells exposed to heat shock treatment. Interestingly heat shock induced high expression of HLA-G6 transcript in JAR cells. Heat shock treatment had no effect on alternative splicing of constitutively expressed HLA-G mRNA in choriocarcinoma cell line JEG-3. HLA-G1 protein expression was induced in JAR and RAJI cell lines by 5-aza-2´- deoxycytidine. In agreement with the differences in the levels of HLA-G transcripts JAR cells express more of HLA-G1 protein than RAJI cells.

Key words: HLA-G; demethylation; histone deacetylation; hypoxia; heat shock

Human leukocyte antigen G (HLA-G) is a nonclassical major histocompatibility class I molecule characterized by a limited polymorphism and tissue distribution restricted to the fetal–maternal interface in extravillous cytotrophoblasts, amnion and in immuneprivileged sites, including thymus and cornea. HLA-G antigen exhibit immunotolerant properties such as inhibition of natural killer (NK) cytolysis and cytotoxic T lymphocyte (CTL) responses. As HLA-G antigen can be expressed in tumor cells it may play an important role in their immune escape mechanisms [1–4].

The primary transcript of *HLA-G* is alternatively spliced into seven mRNAs, which might be translated into seven HLA-G protein isoforms. In addition to full length HLA-G1 protein isoform, HLA-G2 lacking the α2 domain, HLA-G3 devoids of the α2 and α3 domains, and HLA-G4 without the α3 domain are also considered to be membrane bound protein isoforms. Soluble forms of HLA-G can be encoded by HLA-G mRNA containing intron 4 (HLA-G5 and HLA-G6) or intron 2 (HLA-G7). HLA-G6 isoform differs from HLA-G5 isoform by loss of exon 3.

The mechanisms of *HLA-G* gene regulation differ from those of classical *HLA class I* genes because almost all known regulatory sequences for classical *HLA class I* genes are disrupted. Recent studies demonstrated that regulation of *HLA-G* gene activity involve epigenetic mechanisms. Two of the most studied epigenetic phenomena are DNA methylation and histone tail modification [5–10]. DNA is methylated by DNA methyltransferases at the 5-position of the cytosine ring, almost exclusively in the context of CpG dinucleotides. Low levels or a lack of DNA methylation in the promoter region is correlated with active gene expression. DNA is wrapped around a core of eight histones to form nucleosomes, the smallest structural unit of chroma-

Abbreviations: β2m – β2-microglobulin; RT-PCR – reverse transcriptase-polymerase chain reaction; AZA – 5-aza-2´- deoxycytidine; DFX – desferrioxamine; VA – valproic acid; NaBu – sodium butyrate; HS – heat shock; HDAC – histone deacetylase

tin. The basic amino terminal tails of histones protrude out of the nucleosome and are the subject of posttranslational modification, including acetylation by histone acetyltransferases. These modifications influence how tightly or loosely the chromatin is compacted, and thereby plays a regulatory role in gene expression. Most notably, the acetylation of lysine residues on histones H3 and H4 is correlated with active or open chromatin, which allows various transcription factors access to the promoters of target genes. By contrast, deacetylation of lysine residues results in chromatin compaction and inactivation of genes [11, 12].

By treatment of some cell lines with demethylating agent 5-aza-2´- deoxycytidine HLA-G mRNA expression dramatically increased, nevertheless this increase was not always accompanied with induction of HLA-G protein synthesis. These data strongly suggest that *HLA-G* is silenced in different cell lines as a result of CpG site hypermethylation within a 5' regulatory region encompassing 450 bp upstream of the start codon, whereas it is activated upon demethylation. Cell exposure to histone deacetylase inhibitors as sodium butyrate or trichostatin resulted in the induction of HLA-G transcription in only the M8 human melanoma cell line. Although the number of cell lines examined is low it is possible that HDAC-mediated repression of the *HLA-G* gene is not as common as CpG hypermethylation [9].

Epigenetic processes might be dependent on microenvironment conditions, particularly on stress. According to published data HLA-G might be considered as a stress-inducible gene. Importantly hypoxia is a physiologically relevant tumor-related stress that is a common feature of rapidly growing malignant tumors and their metastases [13, 14]. Treatment of melanoma cell lines M8 or 1074mel with hypoxia mimetic desferrioxamine induced HLA-G transcription. *HLA-G* gene responsiveness to hypoxia might be restricted to some melanoma or a few tumor cell types. Interestingly, hypoxia is not the unique stressful condition reversing *HLA-G* gene repression in the M8 melanoma cell line, because transcriptional activity was observed also following heat shock at 42°C or arsenite treatments [15–17].

Epigenetic drugs, whether demethylating agents or histone deacetylase inhibitors target aberrantly heterochromatic regions, leading to reactivation of tumor suppressor genes and/or other genes that are crucial for the normal functioning of cells. Among the genes reactivated by epigenetic drugs are genes that increase immunogenicity of tumor cells. These epigenetic drugs are currently undergoing massive clinical testing for cancer treatment. [18–20]. However, the use of methylation inhibitors or inhibitors of HDAC in cancer therapy might activate the HLA-G molecule of immune tolerance, providing tumor immune escape. Thus, it is critically important to understand the mechanisms involved in the epigenetic changes of the *HLA-G* gene. In the present study, these aspects are re-examined including application of new histone deacetylase inhibitor and hypoxia mimetic chemical.

Material and methods

Cell cultures. Human choriocarcinoma cell lines JEG-3 and JAR (ATCC, Rockville, MD) were maintained in DMEM medium. The following human leukemia cell lines were grown in RPMI 1640 medium: Burkitt's B lymphoma Raji, lymphoid B cell line RPMI 8866 (further on 8866), erythroleukemia cell line K562 and HLA-G transfectants K562-G1 and K562-G2. Media were supplemented with 2mM L-glutamine, 200 μg/ml gentamicin, 0.125 μg/ml amphotericin B and 10% heat-inactivated fetal bovine serum. The transfectants K562-G1 and K562-G2 were kindly provided by Dr. Weiss (Munich, Germany).

Cell treatments. Heat shock treatment of cells was performed at 42°C for 2 hours. Following this stress cells were recovered at 37°C for 6 or 12 hours. Histone deacetylase inhibitory treatments were carried out for 24 hours with 3 mM sodium butyrate (Sigma) or with 5mM valproic acid (NP Biochemicals, LLC). Demethylating treatment with 5-aza-2´-deoxycytidine (Sigma) was carried out for different periods of time (1, 2 or 3 days) at final concentrations of 1, 10 or 100 μM. To analyze the effect of hypoxia cells were treated for 24 hours with hypoxia mimetic desferrioxamine (Sigma) at concentration of 250 μM or with 100 μM $CoCl_2$.

Monoclonal antibodies. Monoclonal antibody (mAb) 4H84 recognizing free heavy chain of all HLA-G protein isoforms was used. This antibody was a generous gift from Dr. McMaster (San Francisco, CA, USA). Monoclonal antibody 87G specific for intact HLA-G antigens was kindly provided by Dr. Geraghty (Seattle, WA, USA).

Flow cytometry. HLA-G cell surface expression was determined by flow cytometry with the 87G monoclonal antibody, which recognizes intact HLA-G molecules associated with β2m. Cells were first incubated with 87G mAb for 1 hour at 4 °C, then washed and subsequently stained with FITC-conjugated rabbit anti-mouse IgG (Dako, Hamburg, Germany) for 30 min at 4 °C. Cytometric analysis was performed on a Coulter Epics Altra Flow Cytometer (Becman Coulter, Florida, USA). The live cells were gated using forward and side scatter and staining with propidium iodide.

Protein extraction and western blotting. 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 (1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin and 0.3 μM aprotinin). Cell extracts were boiled in SDS-PAGE reducing sample buffer and separated on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, USA) and blocked with PBS containing 5% non-fat powder milk. After blocking, membranes were washed in PBS and then incubated with mAb 4H84. The blots were then washed in PBS containing 0.1% Nonidet P-40 and incubated with peroxidase-conjugated rabbit anti-mouse IgG antibodies (Dako, Hamburg, Germany). The membranes were subsequently washed with 0.1 % NP40 in PBS and immu-

noreactive bands were visualized with chemiluminescence reagent (ECL Detection System Santa Cruz Biotechnology, Inc., Santa Cruz,USA).

RNA isolation and semiquantitative RT-PCR analysis. Total RNA was extracted from 5-10 x 10^6 cells using the Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, the cell pellet was lysed in 1000 µl of Trizol and then centrifuged at 12,000 g for 10 min. The supernatant was mixed with 200 µl of chloroform, centrifuged again, and total RNA was precipitated from the aqueous phase with 500 µl isopropyl alcohol. The RNA pellet was washed with 75% ethanol, air-dried, and resuspended in 20 µl diethyl pyrocarbonate (DEPC) treated water. Specific amplification of HLA-G transcripts was performed on cDNA. cDNA was prepared from 2 µg of total RNA by reverse-transcription (RT) in a 20 µl reaction volume using Taq Man Reverse Transcription Reagents (Applied Biosystems): 1x PCR buffer II, 1 mM of each dNTP, 5 mM $MgCl_2$, 10 U RNase inhibitor, 50 U MultiSribe reverse transcriptase, and 2.5 µM oligo $d(T)_{16}$. The reaction was carried out at 42 °C for 60 min, and stopped by heating at 95 °C for 5 min. PCR was performed in total volume of 30 µl containing 6 µl of RT product, 4 mM $MgCl_2$, 0.75 U of AmpliTaq DNA polymerase or AmpliTaq Gold DNA polymerase (Applied Biosystems), 200 µM of each dNTP, and 7 pM of each primer. The following set of primers was used: G.257F (exon 2; 5'-GGA AGA GGA GAC ACG GAA CA) and G.1004R (exon 5 and exon 6 junction; 5'-CCT TTT CAATCT GAG CTC TTC TTT) [21]. As an internal control the ß-actin gene amplification was carried out for each sample using following primers: BGU; 5'-ATG TTT GAG ACC TTC AAC AC and BGL; 5'-CAC GTC ACA CTT CAT GAT GG. The PCR conditions were 5 min at 95 °C for AmpliTaq DNA polymerase or15 min at 95 °C for AmpliTaq Gold DNA polymerase activation, followed by PCR cycles of 1 min at 94 °C, 1 min and 30 sec at 62 °C, and 2 min at 72 °C, with a final extension at 72 °C for 7 min. The thermal cycler used was Perkin Elmer 9600. PCR product accumulation was measured during the exponential phase and the following numbers of PCR cycle were 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 seqence located between PCR primers, were used. HLA-G specific primers and probe were selected to amplify all alternative forms of HLA-G transcripts (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers and probe were used as endogenous control (Table 1), [22].

Table 1. Primers and probes used for TaqMan real-time RT-PCR expression analysis

Name	Sequence
GAPDH forward primer	5'-CATGGGTGTGAACCATGAGAA-3'
GAPDH reverse primer	5'-GGTCATGAGTCCTTCCACGAT-3'
GAPDH probe	5'-AACAGCCTCAAGATCATCAGCAATGCCT-3'
HLA-G forward primer	5'-CTGGTTGTCCTTGCAGCTGTAG-3'
HLA-G reverse primer	5'-CCTTTTCAATCTGAGCTCTTCTTTCT-3'
HLA-G probe	5'-CACTGGAGCTGCGGTCGCTGCT-3'

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 or untreated 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.

Sequencing of HLA-G fragment. DNA fragment 620 bp was excised from the agarose gel and purified using FavorPrep™ GEL Purification Kit (Favorgen Biotech Corp.). Sequencing was performed in both directions using the same amplification primers (G.257F, G.1004R) as sequencing primers by Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) on an ALFexpress II DNA Analysis System (Amersham Biosciences). Sequencing results were compared with the Ensembl Genome Browser database, gene ENSG00000204632 (transcript ENST00000360323).

Results

Increasing sensitivity of the RT-PCR for detection of HLA-G transcription. Previously we and others published that in many cell lines no HLA-G transcripts can be detected [7, 23]. We used in such analyses AmpliTaq DNA polymerase and obtained no evidence for HLA-G transcription as documented for choriocarcinoma JAR and lymphoblastoid 8866 cell lines on Fig. 1A. In order to increase sensitivity of our RT-PCR procedure we decided to use rather AmpliTaq Gold DNA polymerase. This enzyme is provided in an inactive state and requires thermal activation. The hot start of PCR amplification provides increased sensitivity and specificity. As shown in Fig. 1B HLA-G transcripts can be detected in JAR or 8866 cells by RT-PCR with AmpliTaq Gold DNA polymerase. Similarly in some other hematopoetic cell lines initially estimated as negative (MT1, BALL, HL60 and JOK) low HLA-G transcription was also detected with RT-PCR using AmpliTaq Gold DNA polymerase (results not shown). As no HLA-G transcription was detected with RT-PCR using AmpliTaq Gold

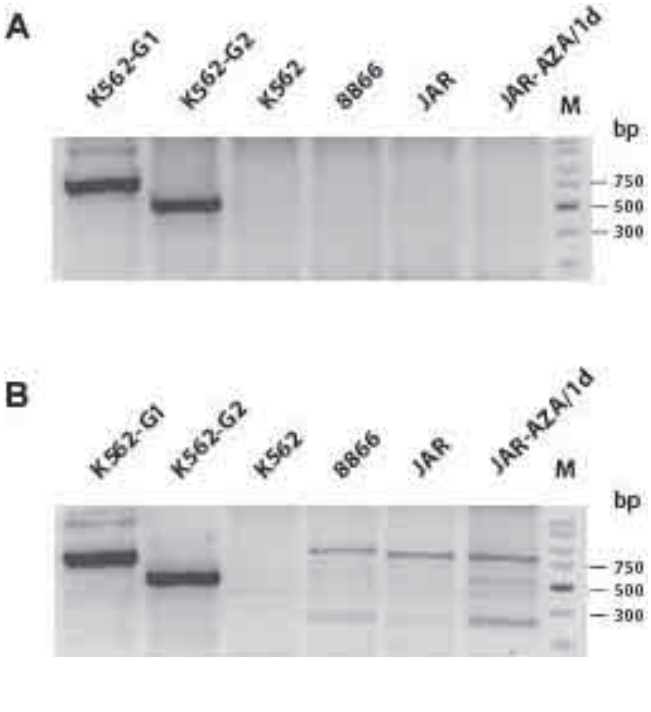

Figure 1. RT-PCR analysis of HLA-G transcripts using two different AmpliTaq DNA polymerases.
Semiquantitative RT-PCR analysis of HLA-G mRNA expression in human cells was performed according the procedure described in Material and methods. In PCR reaction AmpliTaq DNA polymerase (A) or AmpliTaq Gold DNA polymerase (B) was used. Transfectants K562-G1 and K562-G2 are HLA-G positive controls. JAR-AZA/1d: JAR cells treated with 100 μM 5-aza-2´-deoxycytidine for 1 day. M: DNA markers.

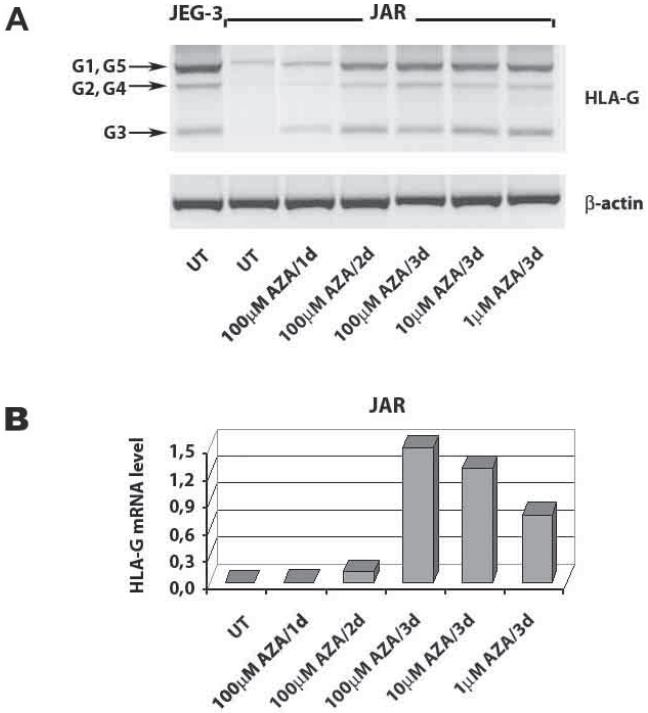

Figure 2. Analysis of HLA-G mRNA expression in choriocarcinoma cell line JAR treated with 5-aza-2´- deoxycytidine (AZA).
JAR cells were treated for 3 days with increasing concentrations of 5-aza-2´- deoxycytidine (1, 10,100 μM) or with 100 μM 5-aza-2´-deoxycytidine (AZA) for different time intervals (1, 2, 3 days). UT: untreated JAR cells. JEG-3 cells (untreated) were used as a HLA-G positive control. HLA-G transcripts were analyzed by RT-PCR (A) or real-time RT-PCR (B). In real-time RT-PCR the HLA-G mRNA levels in JAR cells are compared to JEG-3 cells (assigned a value of 1).

DNA polymerase in K562 cell line (Fig. 1B, and many other cell lines not shown here) we consider detection of HLA-G transcripts under these conditions as highly specific.

Selecting optimal conditions for 5-aza-2´- deoxycytidine activation of HLA-G transcription. To investigate the impact of demethylating treatment on the HLA-G transcription we first carried out RT-PCR analysis of JAR cell line previously treated by 1, 10 or 100 μM concentration 5-aza-2´-deoxycytidine for 1, 2 or 3 days. Total RNA was extracted from both treated and untreated JAR cells and from untreated JEG-3 choriocarcinoma cell line used as a positive control for HLA-G expression. The Fig. 2 shows that 3 days treatment of cells with 100 μM concentration of 5-aza-2´-deoxycytidine resulted in the highest activation of HLA-G transcription in JAR cell line. To visualize the alternative mRNA-forms, HLA-G transcripts were analyzed by semiquantitative RT-PCR (Fig. 2A). The quantities of HLA-G transcripts were evaluated in comparison to those of the JEG-3 cell by using real-time RT-PCR (Fig. 2B). These results show that for highest activation of HLA-G transcription cells must be exposed for three days to 100 μM 5-aza-2´-deoxycytidine. Accordingly in the next experiments cells were treated for three days with 100 μM 5-aza-2´- deoxycytidine.

Activation of HLA-G transcription by methylation inhibitor, histone deacetylation inhibitors and by stress. Next we decided to compare the effect of methylation inhibitor (5-aza-2´- deoxycytidine) to histone deacetylation inhibitors (sodium butyrate and valproic acid), hypoxia and heat shock on the activation of HLA-G transcription in JAR (Fig. 3) and RAJI cell lines (Fig. 4). To visualize the alternative mRNA-forms, HLA-G transcripts were analyzed by semiquantitative RT-PCR (Fig. 3A and 4A). The quantities of HLA-G transcripts were evaluated in comparison to those of the untreated JAR or RAJI cell lines by using real-time RT-PCR (Fig. 3B and 4B). The Figs. 3 and 4 show that the highest activation of HLA-G transcription was achieved by treatment of both cell lines, JAR and RAJI, with demethylating agent 5-aza-2´- deoxycytidine. Treatment of cells with histone deacetylase inhibitors sodium butyrate or valproic acid, also enhanced HLA-G transcription. Nevertheless increase in HLA-G expression by histone deacetylase inhibitors was low as compared with the effect of 5-aza-2´- deoxycytidine. In cells exposed to hypoxia mimetic

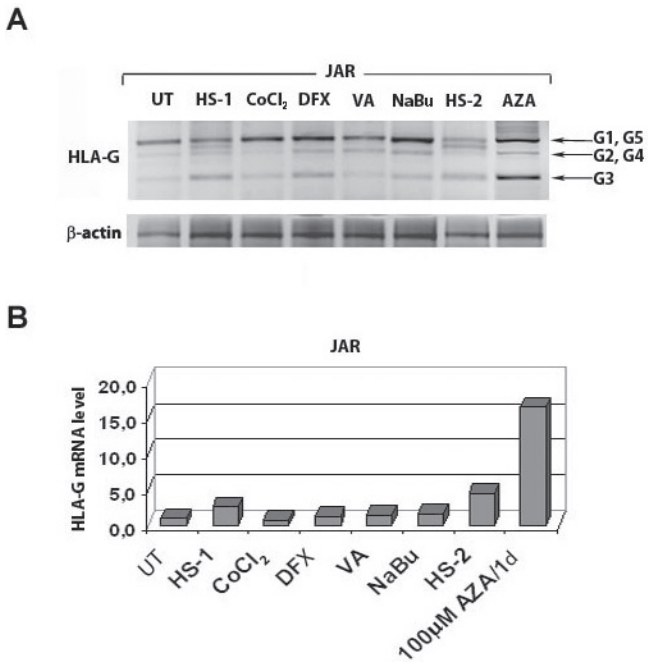

Figure 3. Modulation of HLA-G mRNA expression in choriocarcinoma cell line JAR.
JAR cells were exposed to heat shock (HS) or treated with the following reagents: hypoxia mimitec $CoCl_2$ or DFX (desferrioxamine), histone deacetylase inhibitors VA (valproic acid) or NaBu (sodium butyrate) or demethylating agent AZA (5-aza-2´- deoxycytidine). Heat shocked cells (at 42°C for 2 hours) were allowed to recover at 37° C for 12 hours (HS-1) or for 6 hours (HS-2). UT: untreated JAR cells. HLA-G transcripts were analyzed by RT-PCR (A) or real-time RT-PCR (B). In real-time RT-PCR the HLA-G mRNA levels in treated JAR cells are compared to untreated JAR cells (assigned a value of 1).

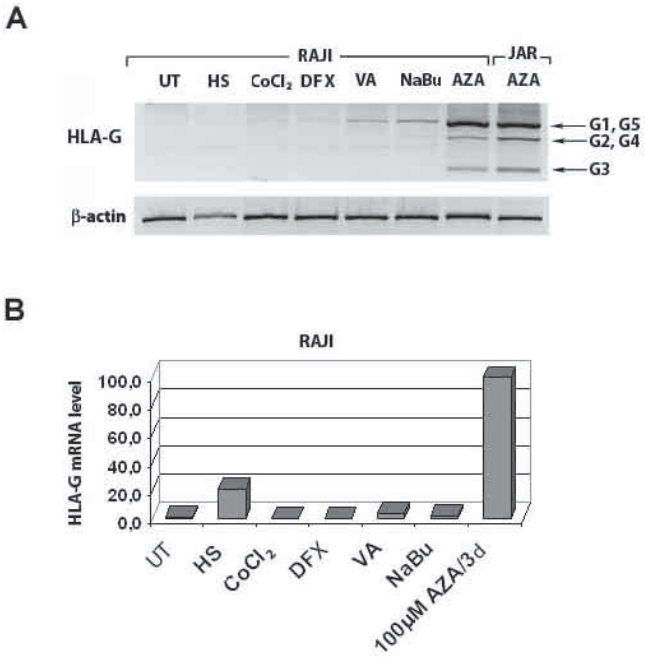

Figure 4. Modulation of HLA-G mRNA expression in lymphoblastoid cell line RAJI.
RAJI cells were exposed to heat shock (HS) or treated with the following reagents: hypoxia mimitec $CoCl_2$ or DFX (desferrioxamine), histone deacetylase inhibitors VA (valproic acid) or NaBu (sodium butyrate) or demethylating agent AZA (5-aza-2´- deoxycytidine). Heat shocked cells (at 42°C for 2 hours) were allowed to recover at 37° C for 6 hours (HS). UT: untreated RAJI cells. HLA-G transcripts were analyzed by RT-PCR (A) or real-time RT-PCR (B). In real-time RT-PCR the HLA-G mRNA levels in treated RAJI cells are compared to untreated RAJI cells (assigned a value of 1).

substances, desferrioxamine or $CoCl_2$ no obvious effect on HLA-G transcription was detected. Heat shock treatment of cells deserves particular attention. In cells exposed to heat shock treatments HLA-G transcription increased to higher extent as after treatment with histone deacetylase inhibitors. Most importantly, following heat shock treatment in JAR cells a pronounced band appeared (Fig. 3A and Fig. 5) Sequencing showed that this band corresponds to HLA-G6 isoform, because there is sequence corresponding to intron 4 but sequence for exon 3 is missing (Table 2). Similar band was not visible in heat shocked RAJI cells. Nevertheless as transcription level in RAJI cells is significantly lower than in JAR cells we cannot exclude the possibility that after heat shock treatment HLA-G6 transcript is also induced. Heat shock treatment had no effect on alternative splicing of constitutively expressed HLA-G mRNA in choriocarcinoma cell line JEG-3 (Fig. 5).

5-aza-2´- deoxycytidine treatment induced HLA-G protein expression in JAR and RAJI cells. To further investigate the impact of enhanced HLA-G transcription on the HLA-G protein expression we carried out flow cytometry analysis of live JAR and RAJI cells and western blot analysis of cell lysates

Table 2. Nucleotide sequence of HLA-G6 transcript isolated from heat shock treated JAR cells

exon 2
.....CGCACAGACTGACAGAATGAACCTGCAGACCCT
exon 4
GCGCGGCTACTACAACCAGAGCGAGGCCAACCCCCC
CAAGACACACGTGACCCACCACCCTGTCTTTGACTAT
GAGGCCACCCTGAGGTGCTGGGCCCTGGGCTTCTAC
CCTGCGGAGATCATACTGACCTGGCAGCGGGATGGG
GAGGACCAGACCCAGGACGTGGAGCTCGTGGAGACC
AGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCA
GCTGTGGTGGTGCCTTCTGGAGAGGAGCAGAGATACA
CGTGCCATGTGCAGCATGAGGGGCTGCCGGAGCCCC
intron 4
TCATGCTGAGATGGA***GTAAGGAGGGAGATGGAGGCA***
TCATGTCTGTTAGGGAAAGCAGGAGCCTCTCTGAAG
ACCTTTAACAGGGTCGGTGGTGAGGGCTGGGGGTCA
exon 5
GAGACCCTCACCTTCACCTCCTTTCCCAGAGCAGTCT
TCCCTGCCCACCATCCCCATCATGGGTATCGTTGCTG
GCCTGGTTGTCCTTGCAGCTGTAGTCACTGGAGCTGC
GGTCGCTGCTGTGCTGTGGA.....

obtained from these cells. Cells were first treated with 5-aza-2´- deoxycytidine at conditions allowing transcriptional

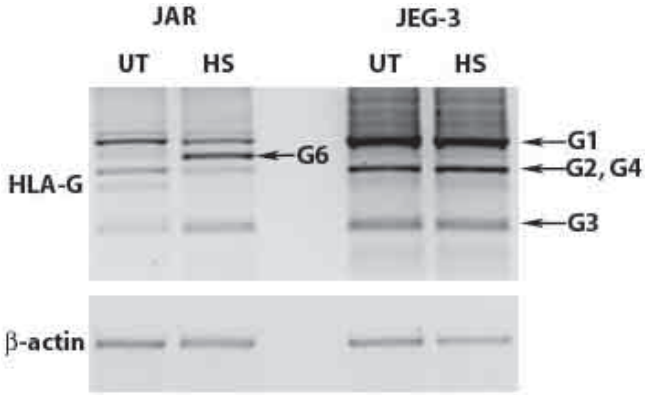

Figure 5. Heat shock-changed HLA-G mRNA alternative splicing in JAR cells.
Heat shock treated choriocarcinoma cell lines JAR or JEG-3 (at 42°C for 2 hours) were allowed to recover at 37° C for 6 hours (HS). UT: untreated JAR or JEG-3 cells. HLA-G transcripts were analyzed by RT-PCR.

activation. Flow cytometry analysis of live JAR cells (Fig. 6A) with 87G mAb showed low cell surface expression of HLA-G protein. Similar analysis showed little if any cell surface expression of HLA-G protein in RAJI cells exposed to 100 μM 5-aza-2´- deoxycytidine (Fig. 6C). No cell surface expression of HLA-G protein was detected in untreated JAR and RAJI cells. The use of the 4H84 mAb which specifically recognizes denatured forms of HLA-protein did not detect any HLA-G proteins in untreated cells, whereas it clearly revealed the presence of HLA-G1 protein isoforms in 5-aza-2´-deoxycytidine treated JAR and RAJI cells (Figs. 6B, 6D). Much lower expression of HLA-G1 protein in RAJI cells as compared with JAR cells is in agreement with the levels of HLA-G transcripts in these cells. Protein expression after other treatments than 5-aza-2´- deoxycytidine was not analyzed as such treatments only slightly increased HLA-G transcription.

Discussion

A number of studies demonstrate that in regulation of *HLA-G* gene transcription are involved epigenetic mechanisms as DNA methylation and histone acetylation [5–10, 15, 16]. Here we re-examined the activation of HLA-G transcription by methylation inhibitor (5-aza-2´- deoxycytidine), histone deacetylation inhibitor (sodium butyrate) and by stress (hypoxia mimetic drug desferrioxamine and heat shock). We also

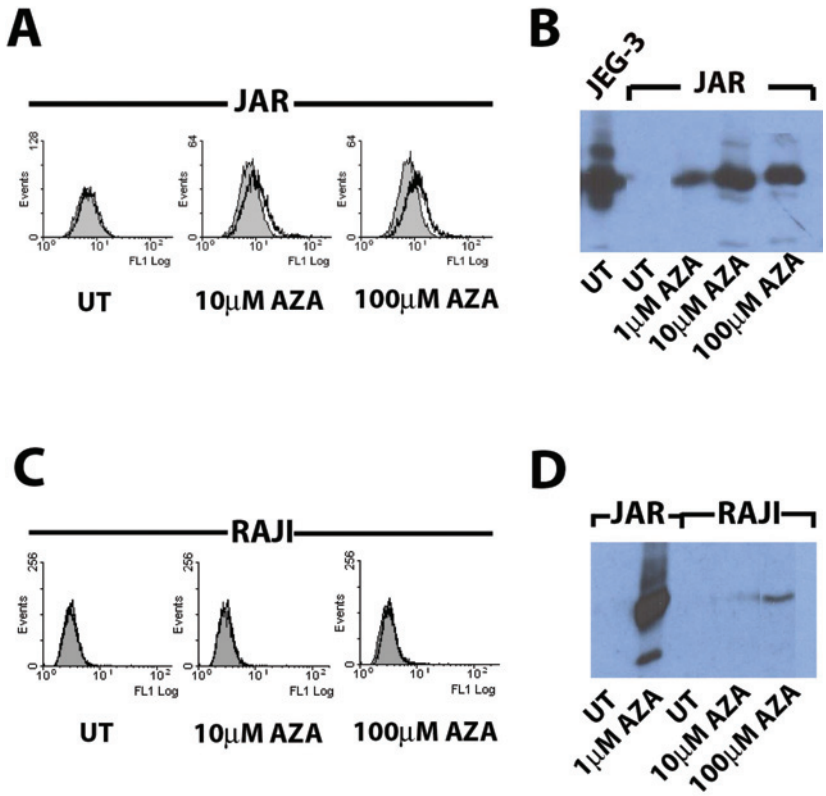

Figure 6. Analysis of HLA-G protein expression in JAR or RAJI cells treated with 5-aza-2´-deoxycytidine (AZA).
Cell lines JAR (A, B) and RAJI (C, D), respectively were treated with 10 or 100 μM AZA for 3 days. UT: untreated JAR or RAJI cells. Expression of cell surface HLA-G proteins was analyzed by flow cytometry using mAb 87G (A, C). Total expression of HLA-G proteins was investigated by western blotting (B, D) using mAb 4H84. Untreated JEG-3 cells were applied as a HLA-G positive control.

included into our experiments another agents as valproic acid (histone deacetylase inhibitor) as well as hypoxia mimetic chemical $CoCl_2$ [24, 25], which were not investigated before. Valproic acid deserves a special attention. Valproic acid alone or in combination with 5-aza-2´- deoxycytidine has significant anti-leukemia activity in humans [18, 19]. These drugs can reactivate tumor suppressor genes and/or other genes that are crucial for successful treatment. However, the use of these epigenetic drugs in cancer therapy might eventually activate the expression of HLA-G antigen, providing tumor immune escape. Therefore it was important to analyze the effect of valproic acid on the *HLA-G* gene transciption.

In all experiments the quantities of HLA-G transcripts were evaluated by real-time RT-PCR. In order to estimate the effect of treatment on individual HLA-G isoforms the products of semiquantitative RT-PCR were also analyzed by gel electrophoresis. We achieved the highest activation of HLA-G transcription by treatment of cells for three days with demethylating agent 5-aza-2´- deoxycytidine at the 100 μM concentration.

Treatment of JAR and RAJI cell lines with histone deacetylase inhibitors (sodium butyrate and valproic acid) also enhanced HLA-G transcription. Nevertheless this increase in HLA-G expression was rather low as compared with activation by 5-aza-2´- deoxycytidine. Hypoxia mimetic substances, desferrioxamine or $CoCl_2$ had no detectable effect on HLA-G transcription. Our results are in a good agreement with other published data, including conclusion that 5-aza-2´-deoxycytidine is so far the most efficient drug for activation of HLA-G gene transcription [6, 9, 10]. Valproic acid itself only moderately activated HLA-G transcription in examined cell lines. In the human therapy valproic acid can be used in combination with 5-aza-2´- deoxycytidine. We were unable to analyze such combination in our experiments as mixture of both drugs was extremely toxic for examined cell lines.

Relatively high increase of HLA-G transcription was detected in cells exposed to heat shock treatments. Interestingly, following heat shock treatment of JAR cells an intensive band appeared, with localization between HLA-G1 and HLA-G2/4 transcripts. According to the sequence analysis this 620 bp band corresponds to HLA-G6 transcript, because it is lacking exon 3 but retaining intron 4. In untreated JAR cell line no HLA-G6 transcript was detected. The same heat shock treatment did not change alternative splicing of constitutively expressed primary HLA-G mRNA in choriocarcinoma cell line JEG-3. Ibrahim et al. also detected HLA-G6 transcript in M8 melanoma cell line following heat shock or arsenite treatment, however he did confirm its identity by sequencing [15]. Change in alternative splicing of primary HLA-G mRNA is not unique event. For example, during long term cultivation of HLA-G positive melanoma cell line Fon, the switch of HLA-G alternative splicing (from cell surface HLA-G1 to intracellular HLA-G2) was described by Rouas-Freiss et al. [26]. Changes in alternative splicing have been reported also in human preimplantation embryos [27]. Up to now little is known about the mechanisms regulating alternative splicing of primary HLA-G mRNA.

Finally, we were interested whether HLA-G protein synthesis is induced by activation of HLA-G transcription. 5-aza-2´- deoxycytidine treatment induced HLA-G protein expression in JAR and RAJI cells. Flow cytometry analysis of live JAR cells showed low cell surface expression of HLA-G protein. Similar analysis showed little if any cell surface expression of HLA-G protein in RAJI cells. Western blot analysis revealed the presence of HLA-G1 protein in JAR and RAJI cells. Much lower expression of HLA-G1 protein in RAJI cells as compared with JAR cells is in agreement with the levels of HLA-G transcripts in these cells. Similar results were described by Mouillot et al. [10]. Other than 5-aza-2´-deoxycytidine activation of HLA-G transcription was not examined for the ability to induce HLA-G protein expression. Nevertheless in future it will be important to find out whether soluble HLA-G6 antigen is produced and released into the medium of heat shock treated JAR cells. Our results further demonstrate that HLA-G transcription and translation can be activated in some cell lines by epigenetic drugs used in therapy of some types of cancer. Therefore the next step must include analysis of HLA-G transcription and HLA-G protein synthesis in tumor cells from patients undergoing therapy with such epigenetic drugs.

This work was supported by the grants 2/5020/25 and 2/6022/6 from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences and by the grant APVV-51-017505 from the Slovak Research and Development Agency.

References

[1] ALGARRA I, CABRERA T, GARRIDO F. The HLA cross road in tumor immunology. Hum Immunol 2000; 61: 65–73.

[2] TAIT BD. HLA class I expression on human cancer cells. Implication for effective Immunotherapy. Hum Immunol 2000; 61: 158–165.

[3] DAVIES B, HIBY S, GARDNER L, et al. HLA-G expression by tumors. Am J Reprod Immunol 2001; 45: 103–107.

[4] ROUAS-FREISS N, MOREAU P, FERRONE S, et al. HLA-G proteins in cancer: Do they provide tumor cells with an escape mechanism? Cancer Res 2005; 65: 10139–10144.

[5] CHANG CC, MURPHY SP, FERRONE S. Differential in vivo and in vitro HLA-G expression in melanoma cells: potential mechanisms. Hum Immunol 2003; 64: 1057–1063.

[6] YAN WH, LIN AF, CHANG CC, FERRONE S. Induction of HLA-G expression in a melanoma cell line OCM-1A following the treatment with 5-aza-2'-deoxycytidine. Cell Res 2005; 15: 523–531.

[7] CHANG CC, FERRONE S. HLA-G in melanoma: can the current controversies be solved? Semin Cancer Biol 2003; 13: 361–369.

[8] CHANG CC, MURPHY SP, FERRONE S. Differential in vivo and in vitro HLA-G expression in melanoma cells: Potential mechanisms. Hum Immunol 2003; 64: 1057–1063.

[9] MOREAU P, MOUILLOT G, ROUSSEAU P, et al. HLA-G gene repression is reversed by demethylation. Proc Natl Acad Sci USA 2003; 100: 1191–1196.

[10] MOUILLOT G, MARCOU C, ROUSSEAU P, et al. HLA-G gene activation in tumor cells involves cis-acting epigenetic changes. Int J Cancer 2005; 113: 928–936.

[11] FUKS F. DNA methylation and histone modifications: teaming up to silence genes. Cur Opin Genet Dev 2005; 15: 490–495.

[12] MINUCCI S, PELICCI PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments of cancer. Nature Rev Cancer 2006; 6: 38–51.

[13] HARRIS AL. Hypoxia- a key regulatory factor in tumor growth. Nature Rev Cancer 2001, 2: 38–47.

[14] YOO CB, JONES PA. Epigenetic therapy of cancer: past, present and future. Nature Rev Drug Discovery 2006; 5: 37–50.

[15] IBRAHIM EC, MORANGE M, DAUSSET J, et al. Heat shock and arsenite induce expression of the nonclassical class I histocompatibility HLA-G gene in tumor cell lines. Cell Stress Chaperon 2000; 5: 207–218.

[16] MOUILLOT G, MARCOU C, ZIDI I, et al. Hypoxia modulates HLA-G gene expression in tumor cells. Human Immunol 2007; 68: 277–285.

[17] NAGAMATSU T, FUJII T, YAMASHITA T, et al. Hypoxia does not reduce HLA-G expression on extravillous cytotrophoblasts. J Reproductive Immunol 2004; 63: 85–95.

[18] YANG H, HOSHINO K, SANCHEZ-GONZALEZ B, et al. Antileukemia activity of the combination of 5-aza-2'-deoxycytidine with valproic acid. Leukemia Res 2005; 29: 739–748.

[19] LUBBERT M. Combined targeting of the epigenetic silence in leukemia: Cooperating activities of DNA methylation and histone deacetylation inhibitors. Leukemia Res 2005; 29: 727–728.

[20] MORA-GARCÍA MdeL, DUENAS-GONZÁLEZ A, HERNÁNDEZ-MONTES J, et al. Up-regulation of HLA class I antigen expression and antigen-specific CTL response in cervical cancer cells by the demethylating agent hydralazine and the histone deacetylase inhibitor valproic acid. J Translant Med 2006; 4: 55–69.

[21] PAUL P, ROUAS-FREISS N, MOREAU P, et al. HLA-G, -E, -F preworkshop: Tools and protocols for analysis of non-classical class I genes transcription and protein expression. Hum Immunol 2000; 61: 1177–1195.

[22] HANIOKA N, OBIKA N, NISHIMURA M, et al. Inducibility of UDP- glucuronosyltransferase 1As by beta-naphthoflavone in HepG2 cells. Food Chem Toxicol 2006; 44: 1251–1260.

[23] POLAKOVA K, BENNINK JR, YEWDELL JW, et al. Mild acid treatment induces cross-reactivity of 4H84 monoclonal antibody specific to nonclassical HLA-G antigen with classical HLA class I molecules. Hum Immunol 2003; 64: 256–264.

[24] GARAYOA M, MARTÍNEZ A, LEE S, et al. Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. Mol Endocrinol 2000; 14: 848–862.

[25] PHIEL CJ, ZHANG F, HUANG, EY, et al. Histone deacetylase is a direct target of valpoic acid, a potent anticonvulsant, mood stabilizer and teratogen. J Biol Chem 2001; 39: 36734–36741.

[26] ROUAS-FREISS N, BRUEL S, MENIER M, et al. Switch of HLA-G alternative splicing in a melanoma cell line causes loss of HLA-G1 expression and sensitivity to NK lysis. Int J Cancer 2005; 117: 114–122.

[27] YAO YQ, BARLOW DH, SARGENT1 IL. Differential expression of alternatively spliced transcripts of HLA-G in human preimplantation embryos and inner cell masses. J Immunol 2005; 175: 8379–8385.