RETRACTED

Induction of HLA-G expression in a melanoma cell line OCM-1A following the treatment with 5-aza-2'-deoxycytidine

Wei Hua YAN[1,2,*], Ai Fen LIN[1], Chien Chung CHANG[2], Soldano FERRONE[2]

[1]*Laboratory Center, Wenzhou Medical College Affiliated Taizhou Hospital, Linhai 317000, China*
[2]*Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*

ABSTRACT

The non-classical HLA class I antigen HLA-G is an immune modulator which inhibits the functions of T cells, NK cells, and the Dendritic cells (DC). As a result, HLA-G expression in malignant cells may provide them with a mechanism to escape the immune surveillance. In melanoma, HLA-G antigen expression has been found in 30% of surgically removed lesions but in less than 1% of established cell lines. One possible mechanism underlying the differential HLA-G expression *in vivo* and *in vitro* is that the HLA-G gene is epigenetically repressed in melanoma cells *in vitro*. To test this hypothesis, we treated the HLA-G negative melanoma cell line OCM-1A with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-AC) and analyzed whether HLA-G expression can be restored. Our data strongly suggest that HLA-G is silenced as a result of CpG hypermethylation within a 5' regulatory region encompassing 220 bp upstream of the start codon. After treatment, HLA-G mRNA expression was dramatically increased. Western blot and flow cytometry showed that HLA-G protein was induced. Interestingly, HLA-G cell surface expression on the 5-AC treated OCM-1A cells is much less than that on the HLA-G positive JEG-3 cells while a similar amount of total HLA-G was observed. Possible mechanisms for the difference were analyzed in the study such as cell cold-treatment, peptide loading and antigen processing machinery components (APM) as well as β_2 microglobulin (β_2-m) expression. Data revealed that the APM component calreticulin might be involved in the lower HLA-G surface expression on OCM-1A cells. Taken together, our results indicated that DNA methylation is an important epigenetic mechanism by which HLA-G antigen expression is modulated in melanoma cells *in vitro*. Furthermore, to the first time, we hypothesized that the deficiency of calreticulin might be involved in the low HLA-G surface expression on the 5-AC treated OCM-1A cells.

Keywords: HLA-G, methylation, 5-aza-2'-deoxycytidine, APM.

INTRODUCTION

The non-classical HLA class I molecule HLA-G was initially discovered on the fetal-maternal interface and considered as a critical mediator in fetal-maternal immune tolerance [1, 2]. Though controversial, studies with strong evidences addressed that HLA-G can also be expressed in tumors [3-5]. HLA-G features a low level of allelic polymorphism and encodes 7 protein isoforms generated by alternatively splicing of primary mRNA, including 4 membrane-bound (HLA-G1 to -G4) and three soluble isoforms (HLA-G5 to -G7) [6]. HLA-G plays key roles in maintenance of immune tolerance by inhibition of NK cells, T cells and DCs via the receptor ILT2, ILT4 and KIR2DL4, providing tumor cells a novel immune escape mechanism to turn off the host immune system [7]. HLA-G expression frequency in cancer has been observed varying according to tumor types, such as melanoma, lung carcinoma, breast carcinoma, clear cell renal carcinoma, colorectal cancer, cutaneous lymphoma. HLA-G expression has also been demonstrated in glioblastoma [8]. An increase of seric HLA-G levels was observed in patients with melanoma. Recently, HLA-G was demonstrated to be a potential marker in malignant ascites of ovarian and breast carcinoma [9, 10]. Among these various human solid tumors analyzed for HLA-G expression, melanoma represents the one in which the largest number of cell lines and surgically removed lesions have been tested. HLA-G expression in melanoma

*Correspondence: Wei Hua YAN
Tel/Fax: +86-576-5226374;
E-mail: yanwhcom@yahoo.com

Abbreviations: HLA (Human leukocyte antigen); 5-AC (5-aza-2'-deoxycytidine); mAb (monoclonal antibody); APM (antigen processing machinery); DC (dendritic cell); β_2-m (β_2 microglobulin).

cell lines in long-term culture and in surgically removed lesions indicated that HLA-G protein expression is higher in lesions than in cell lines, although the HLA-G protein expression frequency is divergent in different reports [11], which suggesting that HLA-G protein expression *in vitro* may require additional *in vivo* stimulators. This possibility is supported by recent report of Malmberg *et al* [12] that HLA-G protein was detected only in freshly isolated ovarian carcinoma cells, but not in cells after long-term culture. The mechanism(s) by which HLA-G expression is modulated in melanoma cells have been investigated, such as stress [13], hypoxia and IFNs [14, 15], leukemia inhibitory factor and glucocorticoids [16, 17]. Others such as epigenetic factors have also been explored in melanoma cells. Epigenetic alterations are widely recognized as contributing to human tumorigenesis. DNA methylation changes are the most frequent epigenetic alterations observed in cancer. Moreau and coworkers reported that 5-AC could slightly induce HLA-G mRNA in a melanoma cell line M8 and cell surface HLA-G expression on 5-AC treated JAR and B-type cell lines [18].

To resolve the issue of differential HLA-G expression between *in vivo* and *in vitro* conditions, we investigated whether the methylation inhibitor 5-AC could induce HLA-G expression in the melanoma cell line OCM-1A. Moreover, possible factors were examined to address why lower surface HLA-G expression on the 5-AC treated OCM-1A cells as compared with that of JEG-3 cells.

MATERIALS AND METHODS

Human cell lines

Human choriocarcinoma cell line JEG-3 (ATCC, Rockville, MD), was maintained in DMEM (Invitrogen, USA). Melanoma cell line OCM-1A was maintained in RPMI-1640 supplemented with L-glutamine (Invitrogen, USA). All cultures were supplemented with 10% heat-inactivated FCS, gentamicin (10 mg/L, Invitrogen) and fungizone (250 mg/L, Invitrogen) and kept at 37ºC in a 5% CO_2 humidified incubator.

Monoclonal antibodies (mAbs)

The following mAbs were used: 4H84, IgG1 anti-denatured HLA-G heavy chain (kindly provided by Dr. McMaster, Department of Stomatology, University of California, San Francisco). MEM-G/09 (Exbio, Prague, Czech Republic), the IgG1 conformational mAb against HLA-G1 and HLA-G5. HLA class I conformational mAb W6/32. HC-10 and L368 was used to detect the classical HLA class I heavy chain and β_2-m in Western blot respectively. The TP25.99 mAb recognizes HLA-A, -B, -C and HLA-E molecules but not HLA-G. mAbs TO-5, TO-2, TO-11, TO-6, SY-1, NOB1, NOB2 and TO-3 were specific for the antigen processing machinery components calnexin, ERp57, calreticulin, LMP2, LMP10, TAP1, TAP2 and tapasin, respectively.

Cell treatments

Demethylating treatment was carried out for d 0, 1, 2, 3, 4 and d 5 with 5-AC (10 mM, Sigma) at final concentrations of 2.5 μM, 5.0 μM, and 10 μM. Melanoma cell line OCM-1A, were cultured for 24 h before the treatment at an initial concentration of 10^4 cells / well in a six-well culture plate (Costar, USA).

Sodium bisulfite genomic sequencing

Genomic DNA was prepared from 1×10^6 cells by lysing for at least 24 h at 37ºC in 5 ml lysis buffer containing 10 mM TRIS-HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl and 0.2% SDS supplemented with 50 μg /ml RNAse A and 100 μg/ml proteinase K (Sigma, USA). Genomic DNA (3 μg) was digested with the restriction enzyme Hind III (Invitrogen, USA), then phenol-extracted, alkali-denatured (NaOH 0.3 M), neutralized (sodium acetate 0.3 M) and precipitated. Bisulfite modification was performed on the denatured DNA by incubating for at least 16 h at 50ºC in 1.2 ml of freshly prepared 2.3 M sodium metabisulfite (pH 5)/0.5 mM hydroquinone (Sigma, USA). DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, USA) according to the manufacturer's instructions. Desulfonation was realized by incubating for 10 min at room temperature in 0.3 M NaOH. DNA was then precipitated with ethanol and 6 M ammonium acetate (pH 7). A 220bp fragment located directly upstream of the start codon of the HLA-G gene was analyzed. PCR amplification of 2 μg DNA was carried out for 1 min at 94ºC, 1 min at 60ºC, 1 min at 72ºC for 35 cycles and a final extension at 72ºC for 5 min using the primers GmF (5'-GATTTAGGGAGATATTGAGATAGAA) and GmR (5'-CCTAATAAAAATAAAAACTAAAACC). PCR fragments were cloned using the pGEM-T Easy Vector System II (Promega, USA). 10 colonies were randomly selected, and plasmids were purified and sequenced. A single methylation specific PCR was performed to test the effectiveness of the sequencing with the following primers. Methylated specific primers: 5'-TTAGGGAGATATTGAGATAGAAC GT and 5'-AATAAAAATAAAAACTAAAACCGCC; and unmethylated specific primers: 5'-TTAGGGAGATATTGAGATAGA ATGT and 5'-CCTAATAAAAATAAAAACTAAAACCACC.

RT-PCR analysis

The choriocarcinoma cell line JEG-3 was used as an HLA-G positive control. Total RNA was extracted from cells with TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. Two micrograms of total RNA was then reverse-transcribed into first-strand cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA) and Oligo(dT)12-18 primers (Invitrogen, USA) in a 20 μl reaction. Subsequently, 1 μl of synthesized cDNA was subjected to PCR in a 50 μl reaction using 2U of Taq DNA polymerase (Roche, Indianapolis, IN) with HLA-G exon 2-specific primer G.257 and 3'-untranslated region-specific primer GA.3U which allowing the detection of all isoforms of alternatively spliced HLA-G mRNA [19]. PCR conditions used were as follows: 94ºC, 1 min; 63ºC, 1.5 min; 72ºC, 2 min for 35 cycles followed by a final extension of 4 min at 65ºC and 4 min at 72ºC. Amplification of β-actin cDNA was carried out in each experiment as an internal control. PCR products were analyzed by electrophoresis in 1.5% agarose gel (Roche, USA), stained with ethidium bromide (Sigma, St. Louis, MO) and visualized on top of a UV light illuminator.

Western blot analysis

Harvested cells were washed three times with cold PBS. Cell pellets were collected and lysed in the lysis buffer (pH7.4, 50 mM Tris-base, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, containing

protease inhibitors PMSF 1 mM) with the final concentration of 1×10^7 cells/ml. After centrifugation at 15,000 *g* at 4ºC for 30 min, supernatants were transferred to Eppenndrof tubes. Cell lysate aliquots of the total proteins from either JEG-3 cells, OCM-1A cells untreated or treated with 5-AC on d 1, 2, 3, 4 and d 5 were separated in 10% SDS-PAGE gel. All samples were heated for 5 min at 95ºC before loading. Proteins were then electroblotted onto PVDF membranes (Millipore, USA) and blocked by incubation with PBS containing 5% non-fat dry milk for at least 4 h. After blocking, membranes were washed in PBS containing 0.2% Tween-20, three times and then probed with the corresponding mAb overnight at 4ºC and washed in PBS containing 0.2% Tween-20 three times. The membranes were subsequently incubated for 30 min at room temperature with Peroxidase-conjugated Affinipure Goat Anti-Mouse IgG Fc fragment (Dako, USA), washed thoroughly with 0.1% Tween-PBS. Finally, membranes were treated with enhanced chemiluminescence reagent (ECL Plus Western Blotting Detection Systems, Amersham Biosciences, USA) and exposed to Biomax film (Kodak, USA) for 1–3 min. To evaluate whether soluble HLA-G secreted into the supernatants, samples from JEG-3 cells, untreated OCM-1A and 5-AC treated OCM-1A cell cultures were collected and subjected to Western blot.

Flow cytometry analysis

HLA-G cell surface expression was determined with the MEM-G/09 mAb (Exbio, Prague, Czech Republic), which recognizes HLA-G molecules associated with β_2m, HLA I antigen was measured with mAb W6/32, and HLA-A, -B, -C, -E but not HLA-G was measured with mAb TP25.99. Second antibody goat anti-mouse R-PE-conjugated IgG1 (DAKO, USA) was used in the experiments. An isotype matched IgG1 mAb MK2-23 was introduced as a negative control. 2×10^6 cells/ml were subjected to flow cytometry analysis (BD, USA).

Peptide-binding assay

Peptides were synthesized by a peptide synthesizer (model 432 A, Applied Biosystems, Georgia University, USA). Three peptides were applied to this study, KIPAQFYIL binds to HLA-G with high affinity, KGPAQFYIL is to a medium extent, and the peptide KGGAQFYIG is irrelative to HLA-G binding which was used as a negative control [20]. Binding assays were performed with the 5-AC treated OCM-1A cells on d 5. Untreated OCM-1A cells served as a HLA-G negative control. Cells were washed twice with FCS free RPMI-1640 media and then incubated for 12 h with the final concentration of 200 μg/ml peptide at 25ºC and 37ºC respectively in 6-well plates with 4×10^6 cells/well. Flow cytometry analysis was performed using MEM-G/09 as the primary antibody and a PE-labelled goat anti-mouse antibody as the secondary antibody. An isotype matched IgG mAb MK2-23 was introduced as a control.

RESULTS

Methylation status of the CpG sites in HLA-G promoter region before and after 5-AC treatment

DNA methylation is an important epigenetic mechanism in gene transcriptional control and may be implicated in HLA-G gene silencing in the HLA-G negative melanoma cell line OCM-1A. In the present study, bisulfite genomic DNA sequencing was performed to assess the methylation status of a 220bp DNA fragment in the HLA-G promoter region before and after the OCM-1A cells exposure to the 5-AC. Data showed that most CpG sites are methylated before the 5-AC treatment (87%), while few methylated sites could be observed after the treatment (2%). The statistically significant difference ($p<0.01$) of the CpG methylation status before and after the 5-AC treatment indicated that CpG hypermethylation resulted in HLA-G repression in the OCM-1A cells *in vitro* (Fig. 1A, B). To confirm the effectiveness of the bisulfite genomic sequencing, a methylation specific PCR (MSP) was introduced and the result is consistent with that of the sequencing (Fig. 1C).

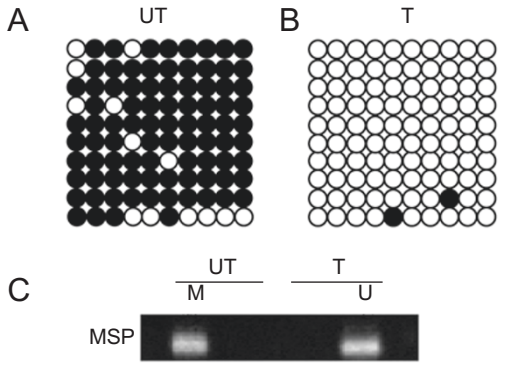

Fig. 1 Methylation status of CpG sites in HLA-G promoter region in 5-AC untreated (UT) or treated (T) OCM-1A cells. **(A, B)** The methylation status of CpG islands of HLA-G in OCM-1A cells before and after the 5-AC treatment was analyzed by bisulfite genomic sequencing. The methylation status of individual CpG dinucleotides is indicated (° and • representing unmethylated and methylated cytosines, respectively). **(C)** Methylation specific PCR (MSP) was performed to confirm the effectiveness of sequencing (U, primers specific for unmethylated CG, M, primers specific for methylated CG).

Dynamic analysis of HLA-G transcription in the OCM-1A cells following the treatment with 5-AC

Various 5-AC concentrations were introduced to treat different types of cell lines as described elsewhere and the effects were shown to be cell type dependent [8, 18]. Our preliminary data showed that the drug was toxic and could lead to cell death. To evaluate the optimal concentration of 5-AC to lessen the toxic effects and to investigate possible epigenetic mechanisms for silencing the HLA-G expression in OCM-1A cells, a dose- and time-dependent study was performed. RT-PCR was carried out to investigate the dynamic changes of HLA-G mRNA expression following the 5-AC treatment at six different time points with three different 5-AC concentrations.

Data showed that HLA-G transcription was induced on d 1 and reached highest on d 4 and then decreased in a manner of time-dependent (Fig. 2). Our preliminary results

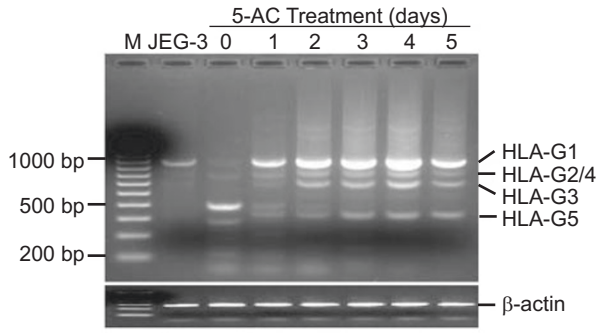

Fig. 2 RT-PCR analysis of HLA-G mRNA expression in the OCM-1A cells treated with 5-AC (2.5 μM). OCM-1A cells treated with 5-AC (2.5 μM) at a particular time point was analyzed with RT-PCR. Total RNA from JEG-3 cells was introduced as an HLA-G positive control and β-actin was amplified as an internal control. M: DNA Marker.

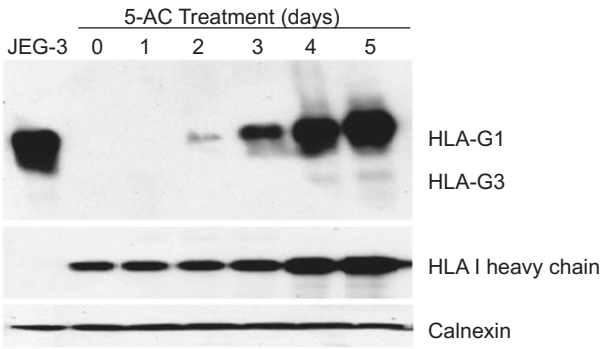

Fig. 3 HLA-G protein expression was induced in the HLA-G-negative OCM-1A cells after 5-AC treatment. Western blot analysis of HLA-G protein expression in the OCM-1A cells was performed with mAb 4H84, which recognizes all denatured HLA-G isoforms (HLA-G1, 39 kD). mAb HC-10 was used to detect the classical HLA class I heavy chain which couldn't recognize HLA-G. Calnexin mAb TO-5 was introduced as an internal control. A similar amount of total HLA-G was observed between the HLA-G positive JEG-3 cells and OCM-1A cells treated with 5-AC on the d 5.

showed that three different 5-AC concentration (2.5 μM, 5.0 μM and 10.0 μM) treatments revealed a similar effect on the HLA-G transcription in the OCM-1A cells, and the final concentration of 2.5 μM was preferred in the following experiments. Dynamic changes of the HLA-G transcriptions were observed with the treatment of 2.5 μM 5-AC, and the appearance of at least HLA-G1 and HLA-G2/4, HLA-G3 and HLA-G5 mRNA forms were presented in this study (Fig. 2).

5-AC treatment induces HLA-G protein expression in OCM-1A cells

To further investigate the impact of 5-AC demethylating treatments on the HLA-G protein expression, we first carried out Western blot analysis of the cell lysates obtained from untreated or OCM-1A cells treated with 5-AC (2.5 μM) at six time points. They were compared with that from the HLA-G positive JEG-3 cells. The application of the mAb 4H84, which specifically recognizes all denatured HLA-G isoforms, did not reveal any HLA-G proteins in untreated OCM-1A cells, whereas it clearly revealed the presence of HLA-G in JEG-3 cells and in the OCM-1A cells exposed to 5-AC on d 2 and reached its highest level on d 5. Besides the HLA-G1, HLA-G3 isoform which exists in the JEG-3 cells could also be observed after d 4 in the 5-AC treated OCM-1A cells (Fig. 3). Western blot results showed that a very similar amount of HLA-G could be observed between the OCM-1A cells treated on d 5 and the HLA-G positive JEG-3 cells. No soluble HLA-G isoform was detected in either untreated or treated OCM-1A cell culture supernatants while this could be seen in the JEG-3 cells, indicating that the dominant isoform induced by 5-AC in the OCM-1A cells is the HLA-G1(Fig. 4).

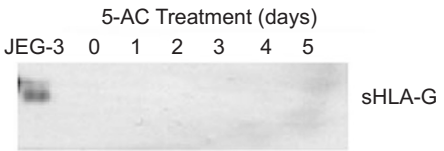

Fig. 4 Western blot analysis of supernatants from both JEG-3 and OCM-1A cell cultures. Cell culture supernatants were subject to Western blot analysis with mAb 4H84. sHLA-G was detected in the JEG-3 cell culture supernatants while this is undetectable in the OCM-1A cells.

Induction of HLA-G protein expression after exposure to 5-AC was also measured by flow cytometry analysis with the mAb MEM-G/09, showing that HLA-G is induced and expressed on the surface of 5-AC treated OCM-1A cells on d 3 and reached highest on d 5 (Fig. 5). Meanwhile, no significant change was found in the HLA class I molecule expression in the OCM-1A cells following the treatment of 5-AC, which is consistent with the results when using mAb TP25.99 to measure the HLA-A, -B, -C and -E but not HLA-G. A reciprocal relationship could be seen in terms of HLA-G and HLA-A, -B, -C and -E expression (Fig. 6). As mentioned above, a similar amount of the total HLA-G expression could be detected both in 5-d 5-AC treated OCM-1A cells and in JEG-3 cells. However, cell surface expression of HLA-G between the treated OCM-1A cells on d 5 and JEG-3 cells was remarkably different. FACS data showed that the HLA-G surface expression on JEG-3

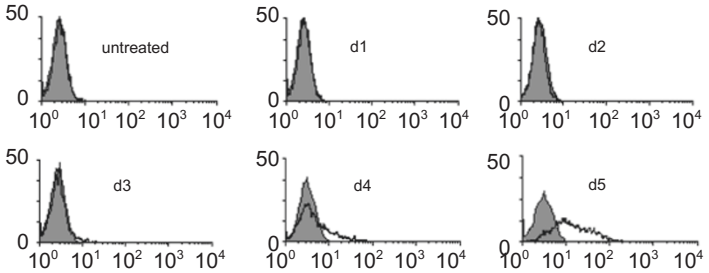

Fig. 5 Induction of HLA-G expression on OCM-1A cells treated with 5-AC. Representative flow cytometry analysis was performed with the anti-HLA-G1/-G5 mAb MEM-G/09 (represented by a white area) and an MK2-23 IgG1 isotype matched antibody (represented by a area in grey). Cell surface expression of HLA-G was induced in OCM-1A cells after the treatment with 2.5 μM 5-AC on d 3 and thereafter.

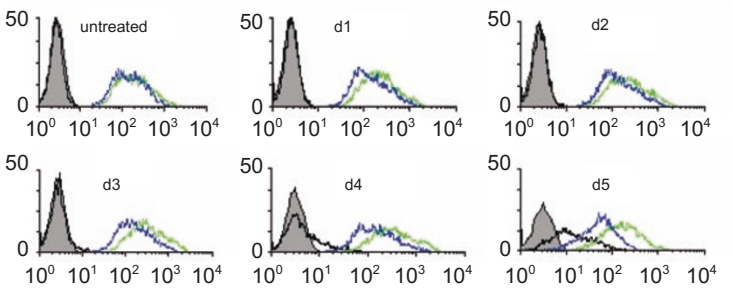

Fig. 6 HLA class I molecule expression on OCM-1A cells treated with 5-AC. HLA-G was measured with mAb MEM-G/09(°), HLA class I was measured with mAb W6/32 (°) and the HLA-A, -B, -C and -E but not HLA-G (°) was measured with mAb TP25.99 respectively. An isotype matched IgG mAb MK2-23 was used as a negative control (•).

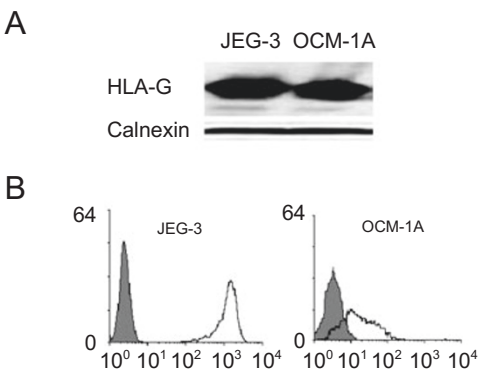

Fig. 7 Comparison of the total and cell surface expression of HLA-G between the JEG-3 cells and 5-AC treated OCM-1A cells on d 5. **(A)** Cell lysate was analyzed with Western blot using mAb 4H84. **(B)** Cell surface expression of HLA-G was analyzed with flow cytometry using mAb MEM-G/9 (°), An isotype matched IgG mAb MK2-23 was used as a negative control (•).

cells was much higher than that on the 5-AC treated OCM-1A cells on d 5 (Fig. 7), which was further strengthened by no detectable soluble HLA-G in OCM-1A cell cultures but in the JEG-3 cells.

Cell cold-treatment and peptide is not required to stabilize surface expression of HLA-G on 5-AC treated OCM-1A cells

Here raise a question why HLA-G cell surface expression is obviously discrepant while there was a similar total amount of HLA-G expression between the 5-AC treated OCM-1A cells and JEG-3 cells. One feature of HLA class I molecule related to its ability to bind peptide is the stabilization of surface MHC class I by lower temperature [21]. When TAP-negative cell lines are cultured at reduced temperature (19~33ºC), heavy chain and β_2-m assembly is promoted and results in a higher level of cell surface expression of MHC heavy chain/β_2-m complexes that do not present endogenous peptides and are labile at 37ºC. This feature is common to HLA class I proteins, and therefore provides a useful point to test whether HLA-G surface expression on the treated OCM-1A cells could be enhanced with this measure. In the present study, we treated OCM-1A cells cultured at 25ºC and 37ºC for 12 h respectively. It was apparent that HLA-G expression under the two temperature was same (Fig. 8).

HLA-G molecule requires peptide association for cell surface stabilization and expression. Peptide loading onto the HLA-G protein plays a critical role in controlling the

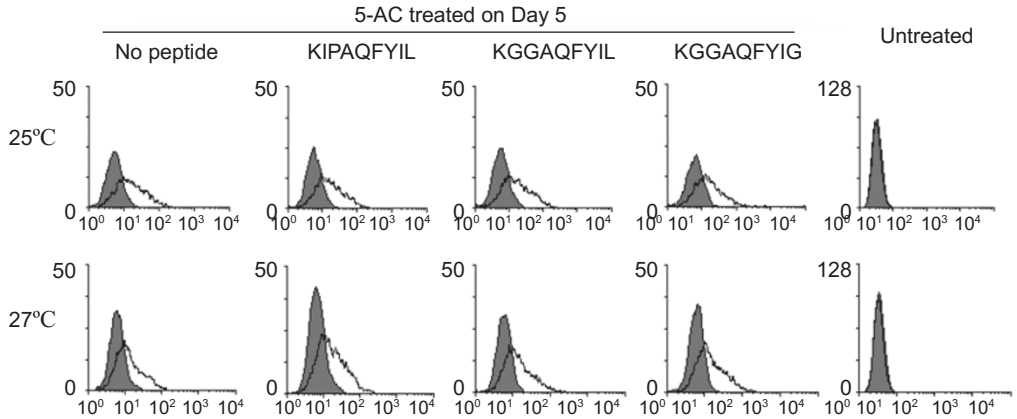

Fig. 8 HLA-G surface expression is unaffected by cold treatment or peptide loading in the OCM-1A cells treated with 5-AC on d 5. Cell surface HLA-G expression was measured by flow cytometry with mAb MEM-G/09 for temperature treatment and different peptide loading. 5-AC treated OCM-1A cells on d 5 were maintained at 37ºC and 25ºC for 12 h respectively. Gray profiles indicate stained with an IgG isotype-matched mAb MK2-23.

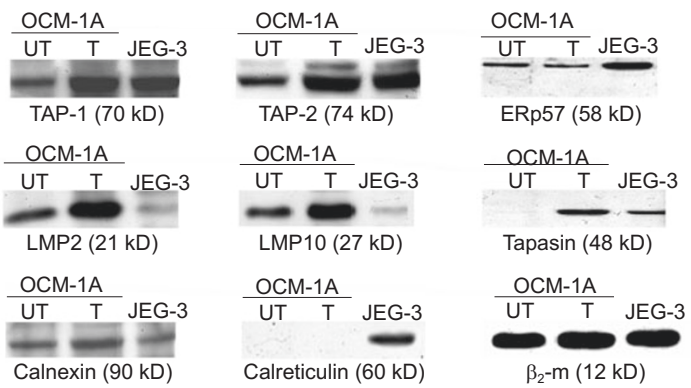

Fig. 9 Western blot analysis of antigen processing machinery components. Aliquots of cell lysates from OCM-1A cells untreated or treated with 5-AC on d 5 and JEG-3 cells were subjected to Western blot with specific APM component mAb. Calreticulin was not observed either in untreated or in 5-AC treated OCM-1A cells on d 5. HLA-G positive cell line JEG-3 was introduced as a control. UT, OCM-1A cells untreated with 5-AC; T, OCM-1A cells treated with 5-AC on d 5.

quality of the molecule reaching cell surface. Previous data indicated that incubation with binding peptides increases cell-surface density of some MHC molecules [22]. Three peptides with different affinity to HLA-G binding were introduced to the 5-d 5-AC treated OCM-1A cells to confirm whether the peptide binding is a determinant factor for HLA-G molecule cell surface stabilization. Exogenous application of peptide KIPAQFYIL has been confirmed to be of high affinity to HLA-G binding, and the peptide KGGAQFYIL to a medium extent. Meanwhile, an HLA-G irrelevant peptide KGGAQFYIG was used as a control. In no case did the HLA-G on the cell surface increase upon incubation with these peptides, as indicated by FACS analysis (Fig. 8).

Calreticulin might be involved in HLA-G cell surface expression on the 5-AC treated OCM-1A cells

Besides cold-treatment and peptide binding, lessons learned from classical HLA class I antigens indicate that folding and assembly of HLA molecules are critical for the regulation of their cell surface expression, which involves intracellular protein complexes and chaperones. The stable assembly of class I molecules with peptides is controlled by a variety of accessory proteins, including chaperones and factors with dedicated roles in class I assembly. Peptide-filled class I molecules are then delivered to the cell surface [22].

As mentioned above, cold-treatment and peptide-loading didn't change the HLA-G cell surface expression on the

5-AC treated OCM-1A cells. We then focus on the antigen processing machinery (APM) components as well as β_2-m whether the deficiency of these factors impair the HLA-G cell surface expression. Data showed that β_2-m was positive in both cells. Meanwhile, eight common components were examined in the 5-AC treated or untreated OCM-1A cells and JEG-3 cells. Result showed that calnexin, ERp57, LMP2, LMP10, TAP1, TAP2 and tapasin exist both in 5-AC treated OCM-1A cells and in JEG-3 cells. However, neither the untreated, nor the 5-AC treated OCM-1A cells was detected for the calreticulin expression, while this component was present in the JEG-3 cells (Fig. 9), indicating that deficiency of calreticulin might be the cause for the low cell surface expression of HLA-G on the 5-AC treated OCM-1A cells.

DISCUSSION

Non-classical HLA class I antigen HLA-G was initially discovered on cytotrophoblast cells invading the maternal endometrium, in that HLA-G was supposed to be a specific modulator for the induction and maintenance of feto-maternal immunotolerance during pregnancy [23]. This hypothesis was supported by the biological function of HLA-G that can inhibit both maternal T cells and uterine NK (uNK) cells, and thereby preventing the semi-foreign fetus from allograft rejection by maternal immune system [24-26]. Initially, HLA-G expression was thought to be limited to cytotrophoblast, thymic. However, more ectopic HLA-G expression in various malignancies was reported, such as melanoma, glioma, renal cell cancer and lung cancer as well as in T cell lymphoma [11, 18]. *In vitro* functional experiments provided evidence for a novel immune escape mechanism of tumor cells by which the immune surveillance through cytotoxic T lymphocytes (CTL) and NK cells is turned off by the expression of HLA-G [27]. Malignant melanoma is the tumor that has been mostly analyzed for HLA-G expression. Comparison of melanoma cell lines in long-term culture and in surgically removed lesions showed that HLA-G protein expression is more frequent in lesions than in cell lines, suggesting HLA-G protein expression *in vitro* may require additional stimulators which modulate translation of HLA-G mRNA. This possibility was proved by Malmberg *et al*.'s investigations [12]. In attempting to explain the differential expression of HLA-G between *in vivo* and *in vitro* conditions, the mechanism(s) by which HLA-G expression is modulated in melanoma cells have been investigated such as cytokines, stress and epigenetic modulations. In the present study, the demethylating agent, DNA methyltransferase inhibitor 5-AC was investigated. Our data showed that the HLA-G silencing in the melanoma cell line OCM-1A was restored in a time-dependent manner following the 5-AC treatment. Three different concentration of the 5-AC were tested with the final concentration of 10.0 μM, 5.0 μM and 2.5 μM respectively, we found that all of them have a similar effect on the induction of HLA-G mRNA expression and the final concentration of 2.5 μM was applied in the current study.

After treatment, a sequential expression of HLA-G mRNA and protein was observed. Induction of HLA-G mRNA expression could be detected on the 5-AC treated OCM-1A cells on d 1 and reached highest on d 4 and then decreased afterwards. Interestingly, the intracellular HLA-G protein expression and cell surface expression was one day and two days behind the mRNA induction respectively, indicating that the HLA-G synthesis and transportation to the cell surface in a manner of phase-dependent way. The work reported here showed that epigenetic mechanisms play a vital role in the regulation of HLA-G expression in the long-term cultured melanoma cells *in vitro*. This could be one of the mechanisms which contribute to the differential expression of HLA-G between the long-cultured melanoma cell lines *in vitro* and the surgical removed lesions *in vivo*. The higher expression of HLA-G *in vivo* may entitle the melanoma to evade host immune attack, suggesting that HLA-G may be an attractive target for melanoma therapy target through epigenetic interference.

Worthy noted, there was markedly different cell surface expression of HLA-G on the 5-d 5-AC treated OCM-1A cells compared with that on the JEG-3 cells. Data showed that cell surface expression of HLA-G was much higher on the JEG-3 cells than that on the treated OCM-1A cells, though both cells have a similar amount of total HLA-G molecule in cell lysates as Western blot analysis revealed. Apart from the cell surface expression of HLA-G, soluble HLA-Gs in cell culture supernatants are important to measure whether HLA-G isoforms other than HLA-G1 are present both in JEG-3 cells and in 5-AC treated OCM-1A cells. Results showed that no HLA-G protein was observed in the OCM-1A cell supernatants but soluble HLA-G does exist in the JEG-3 cell culture supernatants. Based on these data, we concluded that most intracellular HLA-G molecule in the OCM-1A cells was hindered to transport to the cell surface.

To address this differential expression, possible factors including cell cold treatment and peptide loading as well as β_2-m expression were investigated. Previous studies pointed out that incubation with binding peptides and cell cold treatment could increase cell surface density of some MHC molecules [21, 28]. We therefore tested whether this might also be the case with HLA-G on 5-AC treated OCM-1A cells. Three different exogenous peptides were introduced according to previous reports. KIPAQFYIL and KGGAQFYIL are the HLA-G binding peptides with high and medium affinity respectively which could stabilize and

increase the HLA-G cell surface expression [29]. At the same time, an HLA-G irrelative peptide KGGAQFYIG was introduced as a negative control. Our data showed that the density of HLA-G on the cell surface didn't enhance upon incubation with these three peptides or the cold treatment, indicating these factors are not involved in the HLA-G cell surface expression. This seems discrepant with the previous study [29] that these two particular peptides could enhance HLA-G stability on the cell surface. One possible explanation is that before transported to the cell surface, most of the HLA-G without optimal peptides was degraded in ER and thus failed to reach cell surface.

Folding and assembly of HLA molecules are critical for the regulation of their cell surface expression which is controlled by β_2-m and a variety of accessory proteins including chaperones [30, 31]. However, β_2-m could not contribute to the low HLA-G cell surface expression on OCM-1A cells because HLA class I antigens are normally expressed on the untreated OCM-1A cells as the FACS result revealed (Fig. 6), and this was confirmed with β_2-m mAb L383 as Western blot data shown (Fig. 9). We then focused on the antigen processing machinery (APM) components in the regulation of HLA-G cell surface expression. In this study, APM components including TAP1, TAP2, LMP2, LMP10, ERp57, tapasin, calnexin and calreticulin were analyzed in the untreated, 5-d treated with 5-AC OCM-1A cells and the JEG-3 cells. Results indicated that tapasin and calreticulin are absent in the untreated OCM-1A cells. However, TAP1, TAP2, LMP2 and tapasin were increased in the OCM-1A cells following the treatment with 5-AC. To be noted, calreticulin is absent both in untreated and in 5-AC treated OCM-1A cells, while it is highly expressed in the JEG-3 cells. The role of calreticulin in class I assembly was recently evaluated with fibroblast cells derived from mouse embryos that carry a lethal, homozygous mutation in their calreticulin gene [32]. Class I molecules in these cells impaired to load with optimal peptide ligands and thus moderately reduced class I cell surface levels, raising the hypothesis that calreticulin is vital to the HLA-G cell surface expression and this might be the answer to the different cell surface expression of HLA-G between treated OCM-1A cells and JEG-3 cells.

Taken together, our work highlights the epigenetic mechanisms, such as DNA methylation, involved in the modulation of HLA-G gene expression in the melanoma cell line OCM-1A which shed a new light to explain the differential HLA-G expression between the surgical removed lesions and long-cultured cell lines. Furthermore, we hypothesized that the HLA-G cell surface expression is calreticulin dependent in the 5-AC treated OCM-1A cells while the HLA-G binding peptide and cell cold treatment are not involved in the HLA-G cell surface stabilization. The calreticulin restoration is necessary to evaluate whether this application enhances the HLA-G cell surface expression on the 5-AC treated OCM-1A cells.

ACKNOWLEDGEMENTS

We thank Dr. McMaster for the mAb 4H84. This work was supported by Public Health Service (PHS) Grants CA67108 and CA16056 awarded by the National Cancer Institute, U.S. Department of Health & Human Services (DHHS).

REFERENCES

1 Carosella ED, Moreau P, Aracting S, Rouas-Freiss N. HLA-G: a shield against inflammatory aggression. Trends Immunol 2001; **22:**553-5.
2 Fuzzi B, Rizzo R, Criscuoli L, *et al*. HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy. Eur J Immunol 2002; **32:**311-5.
3 Paul P, Rouas-Freiss N, Khalil-Daher I, *et al*. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. Proc Natl Acad Sci U S A 1998; **95:**4510-5.
4 Urosevic M, Kurrer MO, Kamarashev J, *et al*. Human leukocyte antigen G up-regulation in lung cancer associates with high-grade histology, human leukocyte antigen class I loss and interleukin-10 production. Am J Pathol 2001; **159:**817-24.
5 Ibrahim E, Guerra N, Lacombe M, *et al*. Tumor-specific upregulation of the nonclassical class I HLA-G antigen expression in renal carcinoma. Cancer Res 2001; **61:**6838-45.
6 Yan WH, Fan LA. Residues Met^{76} and Gln^{79} in HLA-G α1 domain involved in KIR2DL4 recognition. Cell Res 2005; **15:**176-82.
7 Hofmeister V, Weiss EH. HLA-G modulates immune responses by diverse receptor interactions. Semin Cancer Biol 2003; **13:**317-23.
8 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:**928-36.
9 Singer G, Rebmann V, Chen YC, *et al*. HLA-G is a potential tumor marker in malignant ascites. Clin Cancer Res 2003; **9:**4460-4.
10 Davidson B, Elstrand MB, McMaster MT, *et al*. HLA-G expression in effusions is a possible marker of tumor susceptibility to chemotherapy in ovarian carcinoma. Gynecol Oncol 2005; **96:**42-7.
11 Chang CC, Ferrone S. HLA-G in melanoma: can the current controversies be solved?. Semin Cancer Biol 2003; **13:**361-9.
12 Malmberg KJ, Levitsky V, Norell H, *et al*. IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. J Clin Invest 2002; **110:**1515-23.
13 Ibrahim EC, Morange M, Dausset J, Carosella ED, Paul P. Heat shock and arsenite induce expression of the nonclassical class I histocompatibility HLA-G gene in tumor cell lines. Cell Stress Chaperons 2000; **5:**207-18.
14 Kilburn BA, Wang J, Duniec-Dmuchowski ZM, *et al*. Extracellular matrix composition and hypoxia regulate the expression of HLA-G and integrins in a human trophoblast cell line. Biol Reprod 2000; **62:**739-47.

15 Yang Y, Chu W, Geraghty DE, Hunt JS. Expression of HLA-G in human mononuclear phagocytes and selective induction by IFN-gamma. J Immunol 1996; **156:**4224-31.
16 Bamberger AM, Jenatschke S, Schulte HM, Loning T, Bamberger MC. Leukemia inhibitory factor (LIF) stimulates the human *HLA-G* promoter in JEG3 choriocarcinoma cells. J Clin Endocrinol Metab 2000; **85:**3932-6.
17 Moreau P, Faure O, Lefebvre S, *et al*. Glucocorticoid hormones upregulate levels of *HLA-G* transcripts in trophoblasts. Transplant Proc 2001; **33:**2277-80.
18 Moreau P, Mouillot G, Rousseau P, *et al*. HLA-G gene repression is reversed by demethylation. Proc Natl Acad Sci U S A 2003; **100:**1191-6.
19 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:**1177-95.
20 Diehl M, Münz C, Keilholz W, *et al*. Nonclassical HLA-G molecules are classical peptide presenters. Curr Biol 1996; **6:**305-14.
21 Ljunggren HG, Stam NJ, Ohlen C, *et al*. Empty MHC class I molecules come out in the cold. Nature 1990; **346:**476-80.
22 Paulsson KM. Evolutionary and functional perspectives of the major histocompatibility complex class I antigen- processing machinery. Cell Mol Life Sci 2004; **61:**2446-60.
23 LeMaoult J, Discorde ML, Rouas-Freiss N, *et al*. Biology and functions of human leukocyte antigen-G in health and sickness. Tissue Antigens 2003; **62:**273-84.
24 Fournel S, Aguerre-Girr M, Huc X, *et al*. Cutting edge: soluble HLA-G1 triggers CD95/CD95 ligand-mediated apoptosis in activated $CD8^+$/cells by interacting with CD8. J Immunol 2000; **164:**6100-4.
25 Lila N, Rouas-Freiss N, Dausset J, Carpentier A, Carosella ED. Soluble HLA-G protein secreted by allo-specific $CD4^+$ T cells suppresses the allo-proliferative response: a $CD4^+$ T cell regulatory mechanism. Proc Natl Acad Sci U S A 2001; **98:**12150-5.
26 Marchal-Bras-Goncalves R, Rouas-Freiss N, Connan F, *et al*. A soluble HLA-G protein that inhibits natural killer cell-mediated cytotoxicity. Transplant Proc 2001; **33:**2355-9.
27 Rouas-Freiss N, Moreau P, Menier C, Carosella ED. HLA-G in cancer: a way to turn off the immune system. Semin Cancer Biol 2003; **13:**325-36.
28 Liw WR, Myers NB, Connolly JM, *et al.* The specific binding of peptide ligand to L^d class I major histocompatibility complex molecules determines their antigenic structure. J Exp Med 1991; **173:**449-59.
29 Park B, Lee S, Kim E, *et al*. The truncated cytoplasmic tail of HLA-G serves a quality control function in post-ER compartments. Immunity 2001; **15:**213-24.
30 Kaer LV. Major histocompatibility complex class I-restricted antigen processing and presentation. Tissue Antigens 2002; **60:**1-9.
31 Lehner PL, Cresswell P. Recent developments in MHC-class-I-mediated antigen presentation. Curr Opin Immunol 2004; **16:**82-9.
32 Gao B, Adhikari R, Howarth M, *et al*. Assembly and antigen-presenting function of MHC class I molecules in cells lacking the ER chaperone calreticulin. Immunity 2002; **16:**99-109.

Editor: Dr. Yu Fang SHI

Retraction notice

I am writing this letter in support of Dr. Soldano Ferrone's withdrawal of the paper entitled, **"**Induction of HLA-G expression in a melanoma cell line OCM-1A following the treatment with 5-aza-2'-deoxycytidine**"**, published in *Cell Research* July 2005 issue, 15(7):523-531, by Wei Hua Yan, Ai Fen Lin, myself and Soldano Ferrone**.** I had never seen the content of the paper before it appeared on the PubMed webpage.

Dr. Yan was under my direction while he stayed in Dr. Ferrone's laboratory. Dr. Yan did not design any of the experiments from which the results were published in the paper, nor did he intellectually contribute to the interpretation and discussion of the data. The manuscript was intentionally submitted by Dr. Yan without the approval of myself and Dr. Ferrone, indicating the unethical nature of his behavior. In this regard, I request an immediate retraction of the paper.

Chien-Chung Chang, Ph.D.

A National Cancer Institute-designated Comprehensive Cancer Center A National Comprehensive Cancer Network Member