Natural Killer Cell Cytotoxicity Against SKOV3 after HLA-G Downregulation by shRNA

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ABSTRACT

Background: HLA-G is a nonclassical HLA class I molecule which, when elevated in tumor cells, is one of the main factors involved in tumor evasion of immune responses including NK and T cells. **Objective:** To evaluate the effect of HLA-G downregulation on NK cell cytotoxicity in tumor cell lines. **Methods:** The expression level of HLA-G was measured by real-time PCR and flowcytometry after transfection of SKOV3 by shRNA.1, which targets specific sequences in exon 4, or shRNA.2, which targets both exons 4 and 6. NK-92MI cell cytotoxicity against transfected or untransfected target cell lines was measured with the lactate dehydrogenase (LDH) release assay. The Jeg-3 cell line was used as a positive control. **Results:** Membrane-bound HLA-G expression levels decreased significantly in both cell lines after transfection with both shRNAs compared to their corresponding untransfected cells ($p<0.05$). Jeg-3 cells were better lysed than SKOV3 cells by NK cells during the first 48 h after transfection with both shRNAs. Compared to untransfected cells, shRNA.1-transfected SKOV3 cells were significantly more lysed by NK cells 24 h post-transfection ($p=0.043$). **Conclusion:** As a clinical approach, HLA-G downregulation with shRNA may be effective in cancer therapy by improving immune cell activation.

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Keywords: HLA-G downregulation, shRNA, SKOV3, NK cell cytotoxicity

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INTRODUCTION

Human leukocyte antigen (HLA)-G is a nonclassical HLA class I molecule with restricted tissue distribution (1). It has lower polymorphism in the coding region than classical HLA class I genes, and encodes seven protein isoforms, four membrane-bound (G1, G2, G3, and G4) and three soluble isoforms (G5, G6 and G7) by alternative splicing of its primary mRNA (2). HLA-G modulates the function of natural killer (NK) cells and T lymphocytes by interacting with immunoglobulin-like transcripts (ILT)2, ILT4 and killer immunoglobulin receptor (KIR)2DL4 (3). In addition to extravillous trophoblast cells, overexpression of HLA-G has been observed in various malignancies (4,5). The occurrence of HLA-G expression in both primary tumors and metastatic lesions, but not in normal cells or regressive tumors, suggests that its expression is associated with tumor escape from immune-mediated mechanisms and cancer progression (6).

Effector cells are strongly inhibited by HLA-G1 dimers on the surface of tumor cells and soluble HLA-G5 dimers in the tumor microenvironment, which have a higher affinity for inhibitory receptors than monomers (7). Trogocytosis of HLA-G from tumor cells to T cells or NK cells renders them unresponsive and prevents their cytotoxic activity (8,9). Because of the ability of HLA-G to inhibit immune responses in the tumor microenvironment, blocking HLA-G interactions with its corresponding receptors is a key goal in efforts to restore immune system functioning. In addition, downregulation of HLA-G by interfering RNA can be effective in tumor immunotherapy (10).

This study was designed to evaluate the effect of HLA-G downregulation by shRNA on NK cell cytotoxicity in tumor cell lines.

MATERIALS AND METHODS

Cell Lines. To select HLA-G expressing cell lines as target cells, HLA-G expression was investigated in several human cell lines including: SKOV3 (ovarian carcinoma cell line), SKBR3, MCF7 and MDA-MB231 (breast cancer cell lines), HepG2 (liver cancer cell line), SW-872 (liposarcoma cell line), LNCaP (prostate cancer cell line), Mehr-80 (lung cancer cell line), SW-480 (human colon cancer cell lines) and U437 (monoblastic leukemia cell line) by flowcytometry using PE-conjugated anti-HLA-G antibody (87G mAb) and mouse IgG2a as the isotype control (eBioscience, San Diego, CA, USA). Jeg-3 (choriocarcinoma cell line) was used as positive control (11).

Because of high expression of HLA-G on SKOV3 cell line, this line was selected as the target cell line for the experiments reported here. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The human NK cell line NK-92MI was used as an effector cell line and grown in RPMI 1640 medium supplemented with 12.5% fetal bovine serum, 12.5% horse serum, 1 mM sodium pyruvate and 1 mM nonessential amino acids. Cultures were kept at 37°C, 5% CO_2 and 90% humidity.

Short Hairpin (sh)RNA Transfection. HLA-G silencing was done with the pKLO.1 vector containing a 21-nucleotide shRNA targeting a specific sequence in exon 4 (shRNA.1: 5'-CCGG-CGGCCAATGTGGCTGAACAAA-CTCGAG-TTTGTTCAGCCACATTGGCCG-TTTTTG-3') or in exons 4 and 6 (shRNA.2: 5'-CCGG-TATGAACAGTATGCCTACGAT-CTCGAG-ATCGTAGGCATACTGTTCATA-TTTTTG-3'). This vector carries a puromycin resistance gene as

a selectable marker for mammalian cell selection (Sigma-Aldrich, St Louis, MO, USA). Transfection of the target cell lines Jeg-3 or SKOV3 was done with 8 µL lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a total volume of 2 mL of culture medium containing of 4×10^5 target cells and 4 µg HLA-G shRNA. Cells were cultured in 5 mL of culture medium without antibiotics for 12 h and then selected in culture medium containing 0.5 µg/mL puromycin. To obtain the optimal concentration of puromycin for the selection of transfected cells, the sensitivity of untreated target cell lines to increasing doses of antibiotic (0.25, 0.5, 0.75 and 1 µg/mL) was determined after 24 and 48 h with a lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche Applied Science, Germany).

Analysis of HLA-G Expression in shRNATransfected Cells. The expression of HLA-G at the mRNA and protein level was evaluated by real-time PCR and flowcytometry, respectively, after 24 and 48 h in transfected cells compared to control plasmid-transfected cells and untransfected cells. Total RNA was extracted from 10^6 transfected and untransfected cell lines with RNXplus (CinnaGen, Tehran, Iran). Samples of RNA were treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was constructed with a standard kit (Thermo Fisher Scientific). The relative expression of HLA-G was determined by real-time PCR in triplicate with SYBR Green (Applied Biosystems, Foster City, CA, USA) and normalized to GAPDH with the following forward (F) and reverse (R) primers (Bioneer, Daejeon, South Korea), F: 5'CCATCATGGGTATCGTTGCT3' and R: 5'GCTCCCTCCTTTTCAATCTG3' for HLA-G; F: 5'GGCTGGGGCTCATTTGCAGG3' and R: 5'AGTTGGTGGTGCAGGAGGCA3' for GAPDH. The PCR conditions were as follows: 95°C for 10 min and then 95°C for 15 s; 57°C for 30 s and 60°C for 1 min for 40 cycles. Melting curve analysis was performed by collecting fluorescence data while increasing the temperature from 60°C to 95°C for 1 min. Relative changes in target gene expression were evaluated with the Pfaffl method (12).

The expression of membrane-bound HLA-G on transfected cells compared to untransfected cells was also examined by flowcytometry.

NK Cell Cytotoxicity Assay. Cytotoxicity of NK-92MI against transfected or untransfected target cell lines was measured in triplicate after 12 h with the LDH release assay according to the manufacturer's instructions at effector to target cell ratio (E:T) 10:1 which we got the best results when examined E:T ratios 20:1, 10:1, 5:1 and 2.5:1. In this study, results were obtained in three independent experiments.

Statistical Analysis. Differences in HLA-G expression at the mRNA and protein levels as well as differences in cytotoxicity between transfected and untransfected target cells were analyzed by ANOVA. All statistical analyses were done with SPSS v. 15, and $p<0.05$ was considered statistically significant.

RESULTS

The expression levels of HLA-G in different cell lines are shown in Figure 1. As shown, compared to Jeg-3 as positive control, SKOV3 expressed this molecule much more strongly than other tested tumor cell lines.

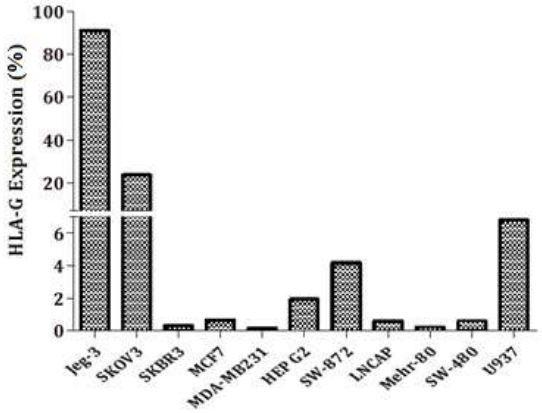

Figure 1. Membrane-bound HLA-G expression levels on different cell lines compared to Jeg-3 as positive control.

Downregulation of HLA-G was observed in both cell lines at the transcription and protein levels after transfection with either shRNA.1 or shRNA.2 ($p<0.05$) (Figure 2).

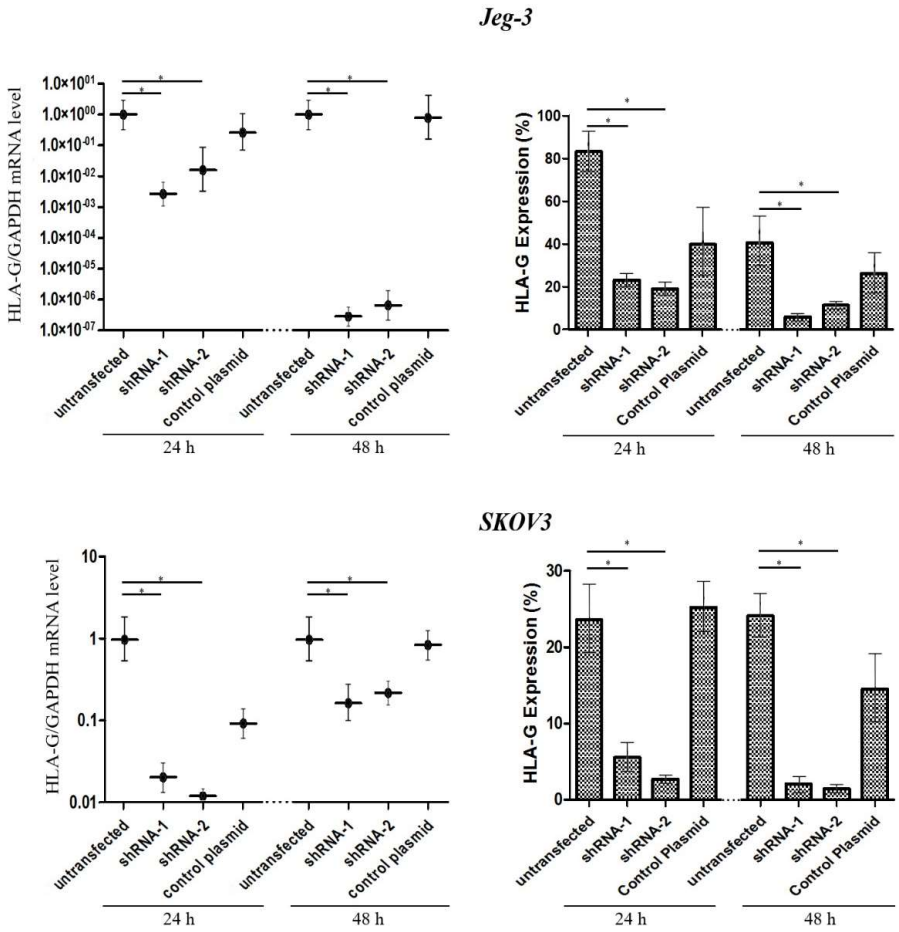

Figure 2. HLA-G expression in mRNA level (left) and protein level (right) in Jeg-3 and SKOV3 cells after transfection with shRNA.1 or shRNA.2 compared to control plasmid-transfected cells and untransfected cells (*shows a significant difference at $p<0.05$).

Unlike SKOV3, which showed maximal downregulation in HLA-G transcripts in the first 24 h (P =0.043) followed by an increasing trend, HLA-G transcription decreased in Jeg-3 during 48 h after transfection (Figure 2- left). The expression of HLA-G molecule on the surface of both cell lines revealed a decreasing trend, however its decline was not remarkable after 48 h compared to 24 h after transfection by either sh-RNAs (Figure 2- right).

NK cell cytotoxicity against target cells transfected with shRNA.1 or shRNA.2 compared to control plasmid-transfected cells and untransfected cells is shown in Figure 3. Membrane-bound HLA-G expression levels decreased significantly in both cell lines after transfection with both shRNAs compared to their corresponding untransfected cells. Jeg-3 cells were better targets for lysis by NK cells during the first 48 h after transfection with both shRNAs. Compared to untransfected cells, only shRNA.1-transfected SKOV3 cells were significantly more lysed by NK cells 24 h post-transfection.

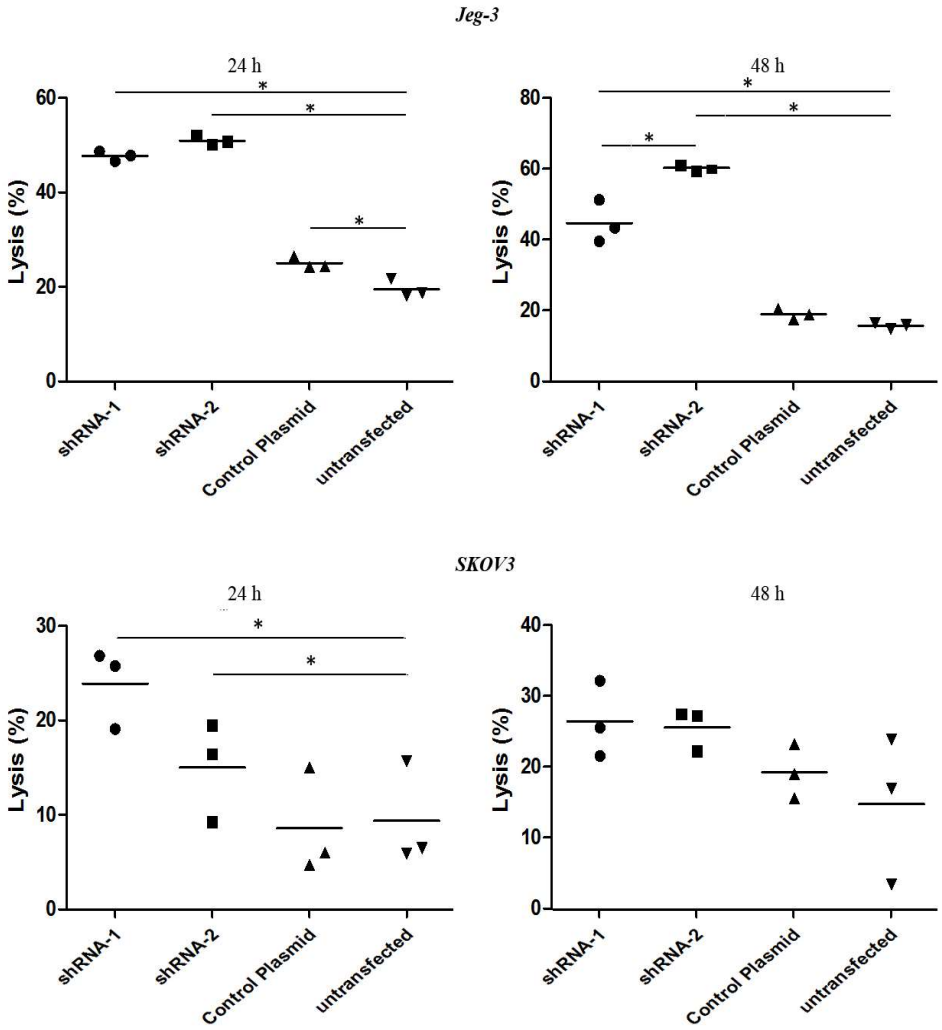

Figure 3. NK-92MI cell cytotoxicity against target cells transfected with shRNA.1or shRNA.2 compared to control plasmid-transfected cells and untransfected cells (* shows a significant difference at p<0.05).

DISCUSSION

Tumor cells evolve multiple mechanisms for immune evasion. Because NK cells and cytotoxic T cells play major roles in tumor rejection, dysfunction of these effector cells due to increased HLA-G expression in tumor cells favors tumor escape from immune attack. In addition to the type of allele, which determines the expression level and receptor affinity (13), HLA-G expression level is regulated by polymorphisms in the promoter and 3' untranslated region (3'UTR) of the gene (14,15). In addition to a 14-bp addition or deletion, there are several single nucleotide polymorphisms (SNPs) in 3'UTR of the HLA-G gene which may affect expression. MicroRNAs (miRs) also play a role in mRNA stability by binding to 3'UTR, which leads to mRNA degradation or translational inhibition. In addition, miR-148a and miR-152 have recently been identified as leading to HLA-G downregulation in the placenta (16). Since miR-148 and -152 regulate various target genes (17) and SNPs in HLA-G 3'UTR may influence the affinity of these miRs for their target sequences, in this study we used shRNA.1 and shRNA.2, which specifically bind to coding regions of the HLA-G gene.

In the tumor microenvironment, epigenetic mechanisms also modulate HLA-G gene expression in tumor cells (18). Therefore, the decreased expression of HLA-G in the tumor cell lines we tested (Figure 1) may be explainable because of repeated *in vitro* culture resulting in diminished or absent signals in the tumor milieu. In this regard, earlier research found reduced or absent HLA-G expression in established cell lines compared to their corresponding fresh tumor lesions (19).

Overexpressed HLA-G in tumor cells and increased levels of soluble HLA-G in the tumor milieu (6) engage not only inhibitory molecules of tumor-infiltrating effector cells, but also induce inhibitory molecules such as ILTs and KIR2DL4 in NK and T cells, which in turn contribute to a robust immune tolerance (20). In addition to the increased secretion of soluble HLA-G isoforms, increased HLA-G1 shedding from the tumor cells also impairs immune surveillance (21).

The discordant HLA-G expression we observed at the mRNA and protein levels (Figure 2) can be explained by the involvement of posttranscriptional mechanisms in regulating HLA-G expression (22). However, a probable limitation of our study was that we did not investigate the presence of soluble HLA-G in culture supernatants.

Our results show that downregulation of HLA-G during the first 48 h after Jeg-3 cell transfection with shRNA resulted in increased lysis of Jeg-3 cells by NK cells compared to untransfected target cells. In this connection, shRNA.2 rendered the Jeg-3 cells more susceptible to lysis by NK cells than shRNA.1 did (Figure 3). However, in contrast to shRNA.1, which targets exon 4, shRNA.2 targets both exons 4 and 6 and may thus be able to silence more HLA-G isoforms. Variable effects of different shRNAs on target gene silencing have been reported previously (23). In addition, certain shRNAs might be processed differently in various target cells, and the same shRNA may show variable effects in different cell lines (24,25). As our results show, the impact of the shRNAs we tested was not the same in Jeg-3 and SKOV3 cells in terms of NK cell-mediated cytolysis (Figure 2).

We found that control plasmid-transfected Jeg-3 cells were significantly more lysed by NK cells 24 h post-transfection compared to untransfected cells, which recovered during the next 24 hours (Figure 3). This might be explained by cell membrane instability arising from vector entry or lipofectamine toxicity (26,27).

Earlier *in vitro* assays in hepatocellular carcinoma cells found that NK cell response was enhanced after HLA-G gene silencing by a pGPU6-GFP-Neo vector containing a 19-nucleotide siRNA that targeted DNA spanning exons 5 and 6 (28). In studies of the protective role of HLA-G in the fetal trophoblast, this part of the HLA-G gene was also targeted by siRNA (11,29).

Apparently, shRNA.1 used in this study silenced HLA-G1, -G2, -G5 and -G6 isoforms after binding to their corresponding target in exon 4, while shRNA.2 bound to both exon 4 and exon 6. HLA-G1, -G2, -G5 and -G6 isoforms silenced after binding to exon 4 and HLA-G1, -G2, -G3 and -G4 isoforms silenced after binding to exon 6. Although isoform G7, which consists only of the α1 domain, was not silenced by either of the shRNAs we tested here, it was able to transduce inhibitory signals through KIR2DL4 in effector cells.

Since multiple microRNAs may control the expression of a certain gene, the combined effect of shRNA.1 and shRNA.2 is likely to be more efficient. Theoretically, targeting nonpolymorphic sequences within exon 2 can be considered potentially useful to silence all isomers. Overall, as a clinical approach, microRNA-mediated HLA-G silencing appears promising in cancer immunotherapy.

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