Soluble HLA-G modulates miRNA-210 and miRNA-451 expression in activated CD4+ T lymphocytes

Fabio Morandi and Vito Pistoia

Laboratory of Oncology, Department of Experimental and Laboratory Medicine, Istituto Giannina Gaslini, Genova 16148, Italy

Correspondence to: F. Morandi, Laboratory of Oncology, Istituto Giannina Gaslini, Largo G. Gaslini 5, Genova 16148, Italy; E-mail: fabiomorandi@ospedale-gaslini.ge.it

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Abstract

In this study, we have investigated the expression of 87 micro (mi)RNAs in activated CD4+ T cells cultured in the presence or absence of the immunoregulatory molecule soluble HLA-G (sHLA-G). We observed (i) a decreased miR-451 expression and (ii) an increased miR-210 expression in sHLA-G-treated CD4+ T cells. By transfecting CD4+ T cells with miR-210 and miR-451 mimics or inhibitors, we found that sHLA-G-mediated modulation of these miRNAs was not related to sHLA-G-mediated inhibition of (i) proliferation and (ii) CXCR3 expression in CD4+ T cells. Finally, we investigated the expression of 14 genes targeted by miR-210 or miR-451 in activated CD4+ T cells, treated or not with sHLA-G. We observed an increased expression of OSR-1 (odd-skipped related 1) and HBP-1 (HMG-box transcription factor 1) and a decreased expression of CXCL16 (chemokine C-X-C motif ligand 16) and C11orf30 (chromosome 11 open reading frame 30) in sHLA-G-treated CD4+ T cells. In conclusion, sHLA-G triggered a modulation of miRNA expression that may in turn modulate downstream gene expression, thus affecting CD4+ T-cell function.

Keywords: CD4+ T lymphocytes, HLA-G, microRNA

Introduction

HLA-G is one of the ‘non-classical’ HLA-class I b molecules, and its main physiological role is the abrogation of maternal NK cell activity against semi-allogeneic fetal tissue, leading to the establishment of immune tolerance at the maternal–fetal interface (1). During the last few years, the role of HLA-G as immunoregulatory molecule in several pathological conditions (i.e. tumors, viral infections and autoimmune disorders) has been extensively described (2, 3).

HLA-G has seven different isoforms that are generated from a single primary transcript through alternative splicing. The expression of membrane-bound molecules (HLA-G1, -G2, -G3 and -G4) and the release of soluble molecules (HLA-G5, -G6, -G7 and soluble HLA-G1, which is generated by shedding from the membrane-bound molecule) have been detected in different cell populations (4). Moreover, soluble (s)HLA-G molecules are present in body fluids (i.e. serum, plasma, cerebrospinal fluid, synovial fluid), and their concentration is often altered in pathological conditions (2, 3).

HLA-G molecules can interact with four different receptors that are expressed on different cell populations: (i) human inhibitory receptors immunoglobulin-like transcript (ILT) 2/CD85j on NK cells, B cells, T cells, monocytes and macrophages, (ii) ILT4/CD85d on monocytes and macrophages, (iii) CD160 on NK cells, T cells and endothelial cells (5) and (iv) killer cell immunoglobulin-like receptor 2DL4 on NK cells and CD8+ T cells (3, 6). The interaction of HLA-G with different receptors leads to different effects on different target cells, that is induction of apoptosis (7), inhibition of cell proliferation (8), cytotoxicity (9), differentiation (10), chemotaxis (11, 12) and modulation of cytokine release (12, 13). sHLA-G has an important role in the control of CD4+ T-cell functions by inhibiting cell proliferation (8), cell cycle progression (14) and activation (15). Moreover, we have recently demonstrated that sHLA-G down-modulates the expression of different chemokine receptors on different T-cell subsets, including CD4+ T cells, leading to the inhibition of chemotaxis of these cells toward their specific ligands (11).

So far, no information is available on possible modulation of micro (mi)RNA expression induced by sHLA-G. miRNAs have been described in the last few years as key modulators of gene expression (16). Moreover, their relevance in the control of the function of different cells of the immune system is constantly increasing (17). In particular, miRNAs have a fundamental role in the control of CD4+ T cell functions, targeting different transcriptional factors (18, 19) that in turn may regulate the expression of several genes, including cytokines (20, 21), membrane-bound (22) and signaling molecules (23) or enzymes (24).

In this paper, we analyzed for the first time sHLA-G-mediated modulation of the expression of different miRNAs that have been...
previously associated with inflammation, leukemia, lymphoma and autoimmune disorders or miRNAs that functionally target specific transcripts encoding cytokines, other immunological regulatory proteins and immune response signaling pathway components. We found that sHLA-G modulated the expression of miR-451 and miR-210 in activated CD4+ T cells. Our results may pave the way to further investigations on a possible sHLA-G-mediated modulation of miRNAs in physiological and pathological conditions.

Methods

HLA-G1/G5 production

Recombinant HLA-G1/G5 protein was produced in the human lymphoblastoid cell line 721.221.G1 (kindly provided by Dr Francesco Puppo, DIMI, Genova) by transfection of the 721.221 parental cell line with human HLA-G1 cDNA (25).

Supernatants were collected from the 721.221.G1 cell line after 72-h culture in RPMI containing 10% fetal bovine serum (RPMI 10% FBS) at 37°C and 5% CO2 and subsequently purified using MEM-G9 mAb and goat anti-mouse beads (Immunotech, Praha, Czech Republic). Soluble HLA-G was quantified by HLA-G1/G5-specific ELISA, as previously described (26).

Cell cultures

The study was approved by the ethical committee of the G. Gaslini Institute, Genova, Italy. Peripheral blood samples were obtained from healthy donors following written informed consent. Mononuclear cells were isolated by Ficoll-Hypaque (Sigma) density gradient.

CD4+ T cells were isolated from peripheral blood mononuclear cells samples using anti-CD4 microbeads (Myltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s protocol. Cells were then washed and cultured for 24 h in RPMI 10% FBS at 37°C and 5% CO2 before being used for further experiments.

Flow cytometry

CD4+ T cells were stained with FITC-conjugated anti-CXCR3 mAb (R&D systems) for 20 min at 4°C and then washed with PBS and 1% FBS (Sigma). The expression of CXCR3 and CD4+ T-cell proliferation (assessed by CFSE dilution) were investigated by running cells on a Gallios cytometer (Beckman Coulter). Data were analyzed using Kaluza software (Beckman Coulter).

RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA or miRNA-enriched RNA preparations were extracted from CD4+ T cells using the miRNeasy Mini kit (Qiagen), following the manufacturer’s procedures. RNA was quantified using a NanoDrop spectrophotometer (NanoDrop products, Wilmington, DE, USA). Total RNA (1 µg) was incubated for 5 min at 42°C with GE buffer (SABiosciences, Frederick, MD, USA) to eliminate genomic DNA contamination and then reverse transcribed using RT2 First Strand Kit (SABiosciences), following the manufacturer’s procedures.

miRNA expression was evaluated in miRNA-enriched RNA preparations using Human Immunopathology miRNA PCR Array (SABiosciences). SNORD44, SNORD47, SNORD48 and U6 were used as housekeeping genes.

The expression of miRNA downstream genes was evaluated on total RNA preparations using a customized RT2 PCR Array (SABiosciences) that included the following genes: PSMB8 (proteasome subunit, beta type, 8), TBC1D9B (TBC1 domain family, member 9B), AIFM3 (apoptosis-inducing factor, mitochondrion-associated 3), SLC25A26 (solute carrier family 25, member 26), ATAD2B (ATPase, AAA domain-containing protein 2B), COL3A1 (collagen, type III, alpha 1), HBP1 (HMG-box transcription factor 1), HMGCN1 (hemicentin 1), OSR1 (odd-skipped related 1), VAMP7 (vesicle-associated membrane protein 7), CXCL16 (chemokine C-X-C motif ligand 16), GRSF1 (G-rich RNA sequence binding factor 1), MEX3C (RNA-binding protein) and C11orf30. GAPDH and C11orf30 (chromosome 11 open reading frame 30) were used as housekeeping genes.

Quantitative real-time PCR (qRT–PCR) analysis was performed following the manufacturer’s protocols, using ABI 7700 (Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: 95°C for 2 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min.

Sequence Detection System software (Applied Biosystems) was used to determine threshold cycle (Ct) values. Average ΔCt values were calculated using PCR Array Data Analysis software v3.3 (SABiosciences) as follows: [Ct (gene of interest) − average Ct (housekeeping genes)]. Results were expressed as 2-ΔΔCt values or fold change values (2-ΔCt) values of CD4+ T cells cultured in the presence of sHLA-G/2-ΔΔCt values of CD4+ T cells cultured in the absence of sHLA-G).

miRNA mimics and inhibitors

In some experiments, CD4+ T cells were transfected with (i) miR-210 mimic, (ii) miR-210 inhibitor, (iii) miR-451 mimic or (iv) miR-451 inhibitor (Qiagen) using HiPerFect Transfection Reagent (Qiagen), following the manufacturer’s protocol. Cells were then washed and cultured for 24 h in RPMI 10% FBS at 37°C and 5% CO2 before being used for further experiments.

Statistics

Statistical analysis was performed using Prism 5.03 software (GraphPad Software). Gaussian distribution of data was analyzed using the Kolmogorov–Smirnov test. The Student’s t-test or Mann–Whitney test was used to compare data, depending on data distribution. The significance range was as follows: *P < 0.05 (significant), **P < 0.005, and ***P < 0.0005.

Results

sHLA-G down-regulated miR-451 and up-regulated miR-210 expression in activated CD4+ T lymphocytes

The expression of 87 human miRNA, which have been previously characterized for their role in immunology and
immunopathology, was evaluated by qRT–PCR on activated CD4+ T cells and cultured in the presence or absence of 100 ng/ml sHLA-G. This concentration was chosen on the basis of a previous study performed on CD4+ T cells (11).

Means of results obtained from six different experiments are summarized in Table 1. The high variability observed among six different experiments may be related to (i) a different activation status of CD4+ T cells in different donors or (ii) an interaction with different environmental factors, such as cytokines, growth factors and so on.

The expression of 11 out of 87 miRNAs was modulated by sHLA-G, with at least a 2.5-fold ratio between treated and untreated cells (these miRNAs are highlighted in Table 1). However, as shown in Fig. 1, panel A, sHLA-G-mediated modulation was statistically significant only for two miRNAs. In particular, sHLA-G significantly (i) down-regulated the expression of miR-451 (2−ΔΔCt value ± SD: untreated −1.24 ± 0.29 versus treated −1.6 ± 0.18; Student’s t-test P = 0.02) and (ii) up-regulated the expression of miR-210 (2−ΔΔCt value ± SD: untreated −1.49 ± 0.6 versus treated −1.05 ± 0.03, Student’s t-test P = 0.05).

**Table 1.** Fold change of miRNAs expression in sHLA-G-treated CD4+ T cells.

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**Modulation of miR-451 and miR-210 expression is not implicated in sHLA-G-mediated inhibition of proliferation and CXCR3 expression in activated CD4+ T cells**

It has been previously demonstrated that sHLA-G (i) inhibits proliferation and (ii) down-modulates expression of different chemokine receptors on CD4+ T cells. We tested whether sHLA-G-mediated modulation of miR-451 and miR-210 expression was related to these effects. CXCR3 expression was evaluated because the expression of this receptor was strongly down-modulated by sHLA-G in mostly T-cell subsets (11).

First, CD4+ T cells were transfected with miR-210 mimic or miR-451 inhibitor, to mimic sHLA-G-mediated modulation. However, as shown in Fig. 1, CD4+ T cells transfected with miR-210 mimic or miR-451 inhibitor did not show any inhibition of T-cell proliferation (panel B) or modulation of CXCR3 expression (panel C).

Next, CD4+ T cells were transfected with miR-210 inhibitor or miR-451 mimic to counterbalance the modulation induced by sHLA-G. Cells were then cultured in the presence or absence of sHLA-G. sHLA-G significantly inhibited proliferation (Fig. 1, panel B, % proliferating cells ± SD: ctr 84.4 ± 2.07 versus sHLA-G 41.1 ± 4.2, Mann–Whitney test P = 0.003) and CXCR3 expression (Fig. 1, panel C, % CXCR3+ cells ± SD: ctr 57.5 ± 4.4 versus sHLA-G 41.1 ± 4.2, Mann–Whitney test P = 0.03) on CD4+ T cells, as previously described. However, neither miR-210 inhibitor nor miR-451 mimic were effective in counteracting the modulation of proliferation (panel A) and CXCR3 expression (panel B) performed by sHLA-G.

**OSR1, CXCL16, C11orf30 and HBP1 gene expression is modulated by sHLA-G in activated CD4+ T cells**

Finally, we have selected 14 top-score downstream genes of miR-210 and miR-451 on the basis of the results obtained with different prediction algorithms (www.mirbase.org, www.mirnablog.com). The selected genes among miR-451 downstream genes were PSMB8, OSR1, TBC1D9B, GRSF1, MEX3C, C11orf30 and HMCN1. The selected genes among miR-210 downstream genes were VAMP7, AIFM3, CXCL16, SL25CA26, ATAD2B, COL3A1 and HBP1. GAPDH and ACTB were tested as housekeeping genes.

We tested the expression of these genes by qRT–PCR in activated CD4+ T cells cultured in the presence or absence of sHLA-G. As shown in Fig. 2, sHLA-G significantly up-regulated the expression of OSR-1 (2−ΔΔCt value ± SD: untreated 0.0014 ± 0.0011 versus treated 0.0056 ± 0.0019, Mann–Whitney test P = 0.03) and HBP-1 (2−ΔΔCt value ± SD: untreated 0.0063 ± 0.0033 versus treated 0.014 ± 0.015, Mann–Whitney test P = 0.03). The expression of CXCL16 and C11orf30 was significantly dampened by sHLA-G treatment (CXCL16 2−ΔΔCt value ± SD: untreated 0.01 ± 0.013 versus treated
Fig. 1. Panel A. Modulation of miRNA expression by sHLA-G. Expression of 87 miRNA was evaluated by qRT–PCR on CD4+ T cells cultured in the presence (black symbols) or absence (white symbols) of sHLA-G (100 ng/ml). Results are expressed as $2^{-\Delta\Delta Ct}$ values. The expression of 11 miRNA, which display at least 2.5-fold change (mean $2^{-\Delta\Delta Ct}$ medium alone/mean $2^{-\Delta\Delta Ct}$ sHLA-G) is shown. Each symbol represents a single experiment, and horizontal bars indicate means. *P values are indicated where the difference is statistically significant (Student's t-test). Panel B. CD4+
Results

0.0056 ± 0.0019, Mann–Whitney test P = 0.01; C11orf30 2−\(\Delta\)Ct value ± SD: untreated 0.0041 ± 0.002 versus treated 0.009 ± 0.01, Mann–Whitney test P = 0.04 (Fig. 2).

Discussion

To our knowledge, we have here provided the first evidence that sHLA-G may modulate the expression of microRNAs in immune effector cells.

In particular, we have reported a modulation of miR-210 and miR-451 expression performed by sHLA-G in activated CD4+ T cells. We have demonstrated that modulation of miR-210 and miR-451 was not functionally related to sHLA-G-mediated inhibition of proliferation or modulation of chemokine receptors expression in CD4+ T cells.

We have analyzed sHLA-G-mediated modulation of different putative downstream genes of miR-451 and miR-210 that have been selected on the basis of different prediction.

Fig. 2. Expression of miR-210 and miR-451 downstream genes. Expression of PSMB8, TBC1D9B, AIFM3, SLC25A26, ATAD2B, COL3A1, HBP1, HMCN1, OSR1, VAMP7, CXCL16, GRSF1, MEX3C and C11orf30 genes was evaluated by qRT–PCR on CD4+ T cells stimulated with anti-CD3 mAb in the presence (gray bars) or absence (white bars) of sHLA-G (100 ng/ml). Results are expressed as 2−\(\Delta\)Ct values. Means of six different experiments ± SD are shown. P values are indicated where the difference is statistically significant (Mann–Whitney test).

T-cell proliferation. CD4+ T cells were stained with CFSE and then treated as follows: (i) CD4+ T cells (a) untransfected (ctr) (b) transfected with miR-210 mimic or (c) transfected with miR-451 inhibitor were stimulated with anti-CD3 mAb; (ii) CD4+ T cells (a) untransfected (ctr), (b) transfected with miR-451 mimic or (c) transfected with miR-210 inhibitor were stimulated with anti-CD3 mAb in the presence of sHLA-G (100 ng/ml). Proliferation was assessed by CFSE dilution, running cells on a flow cytometer. Results are expressed as percentage of proliferating cells. Means of six different experiments ± SD is shown. P values are indicated where the difference is statistically significant (Mann–Whitney test).

Panel C. CXCR3 expression. CD4+ T cells were treated as follows: (i) CD4+ T cells (a) untransfected (ctr) (b) transfected with miR-210 mimic or (c) transfected with miR-451 inhibitor were stimulated with anti-CD3 mAb; (ii) CD4+ T cells (a) untransfected (ctr) (b) transfected with miR-451 mimic or (c) transfected with miR-210 inhibitor were stimulated with anti-CD3 mAb in the presence of sHLA-G (100 ng/ml). CXCR3 expression was evaluated by flow cytometry. Results are expressed as percentage of CXCR3+ cells. Means of six different experiments ± SD are shown. P values are indicated where the difference is statistically significant (Mann–Whitney test).
algorithms. We have found that sHLA-G up-regulated the expression of HBP-1 and OSR-1 and down-regulated the expression of CXCL16 and C11orf30 in CD4+ T cells.

HBP-1 is a transcriptional repressor, whereas OSR-1 is a chloride cotransporter. No information is so far available of their function in CD4+ T cells. However, by up-regulating the expression of a transcriptional repressor, sHLA-G may affect the expression of several downstream genes.

CXCL16 is a chemokine that mediates the homing of CD8+ T cells into human skin, and thereby, it contributes to psoriasis pathogenesis. No information is available on CXCL16 expression and/or release in CD4+ T cells. In principle, sHLA-G-mediated down-modulation of CXCL16 release by CD4+ T cells may contribute to amelioration of psoriasis symptoms. Accordingly, sHLA-G has been previously detected in psoriatic skin and correlated with a better prognosis (27).

The C11orf30 gene, whose expression was dampened by sHLA-G, is a putative target of miR-451 that is also down-regulated by sHLA-G. We can speculate that C11orf30 is an indirect target gene that in turn may be regulated by other transcription factor(s) that are directly targeted by miR-451, as previously described for the COX-2 gene (28). EMSY, which is encoded by the C11orf30 gene, is an oncogenic interacting partner of BRCA2 that functions also as a transcriptional repressor (29). Again, sHLA-G may influence the expression of several downstream genes by modulating the expression of a transcriptional repressor.

In conclusion, sHLA-G-mediated modulation of miR-210 and -451 may have a role in the immunoregulatory properties of this molecule, possibly through the regulation of gene transcription.

Our data warrant further investigations aimed to (i) clarify the role of miRNAs in sHLA-G-mediated immunoregulation, and (ii) analyze a possible modulation of the expression of additional miRNAs performed by sHLA-G. Moreover, additional studies are needed to assess a possible sHLA-G-mediated modulation of miRNA expression in different cell populations.

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Conflict of interest: The authors declare no conflicts of interest.

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References