Binding analysis of HLA-G specific antibodies to hematopoietic cells isolated from leukemia patients*

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Expression of HLA-G on the surface of malignant hematopoietic cells isolated from leukemia patients was analyzed by flow cytometry using monoclonal antibodies (mAbs) recognizing both, intact HLA-G complex (87G, 01G and MEM-G9) as well as HLA-G free heavy chain (4H84, MEM-G/1 and MEM-G/2). Prerequisite of HLA-G detection by mAbs specific to free heavy chain was mild acid treatment, which dissociates intact HLA-G complex. All mAbs, with the exception of 4H84 mAb, did not indicate the presence of HLA-G antigen in leukemia cells. Positive staining with 4H84 mAb was detected in acid-treated cells isolated from 16 out of 30 patients. Intensity of staining increased after IFN-γ pre-incubation in most cases. Immunoblot analyses and RT-PCR, however, failed to detect HLA-G antigen or HLA-G transcripts in cells that bind 4H84 mAb after acid-treatment. The binding of 4H84 mAb can be explained by the acid-induced cross-reactivity of this HLA-G specific mAb with classical HLA class I molecules [15]. The results described here further demonstrate that the HLA-G molecule is not expressed in freshly isolated human leukemia cells.

Key words: HLA-G antigen, human leukemia cells, flow cytometry, immunoblotting, RT-PCR.

HLA-G is a non-classical major histocompatibility class I molecule characterized by a limited polymorphism and expression restricted mainly to the extravillous trophoblasts of human placenta [3]. HLA-G is thought to play an important role in the protection of semiallogeneic fetus from maternal rejection by NK cells. It has been proposed that tumor cells may also express non-classical HLA-G class I antigens [5, 9]. Despite the HLA-G transcriptional activity in some tumor cell lines, the expression of HLA-G protein in tumors remains highly controversial [2, 8, 11, 19, 20, 21].

We have focused our interest on leukemia patients because they have not yet been extensively analyzed for the expression of HLA-G. In previous investigation we failed to detect HLA-G in tumor cell lines of different origin as well as in malignant hematopoietic cells from leukemia patients [14,16]. The present report describes extended analysis of HLA-G expression in malignant hematopoietic cells from leukemia patients. The results described here further demonstrate that the HLA-G molecule is not expressed in freshly isolated human leukemia cells.

Material and methods

Leukemia cells from patients. Peripheral blood or bone marrow samples were obtained from patients with leukemia or lymphoma after informed consent. Diagnosis and staging of disease is based on standard clinical, morphological and immunophenotyping criteria. Characteristics of patients are listed in Table 1.

Mononuclear cells from blood or bone marrow were collected after separation by Ficoll-Hypaque density gradient centrifugation. Freshly isolated cells were used for flow cy-

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Table 1. Characteristics of patients

PIN	Sex/Age	Diagnosis		Source
10	F/26	AML-M2	DN	PB
11	M/38	AML	DN	BM,PB
12	M/52	AML	relapse	BM,PB
13	F/51	AML-M4	DN	BM
14	M/19	AML	DN	BM
15	F/58	AML-M3	DN	PB
16	F/13	AML-M5	DN	BM
17	F/49	AML	DN	BM
18	M/39	AML	DN	BM
19	M/58	AML-M2	parc.remis.	PB
20	F/40	AML	DN	PB
21	M/56	AML-M1	DN	BM,PB
27	M/62	CML	blast.crisis	BM,PB
28	M/11	CML	parc.remis.	BM
29	F/70	CML	blast	PB
30	F/11	ALL	DN	BM
31	M/64	ALL-B	DN	PB
32	M/16	ALL-T	DN	BM
33	F/6	ALL	relapse	PB
34	F/3	ALL	DN	BM
35	M/3	ALL-B	remis.	BM
36	M/22	ALL	relapse	BM
37	F/29	ALL	DN	PB
38	M/58	ALL	DN	PB
44	F/70	CLL-B	DN	PB
46	M/65	CLL-B	DN	PB
47	M/43	HCL	remis.	BM
48	F/59	HCL	remis.	BM
49	F/75	NHL-T	DN	PB
50	M/21	NHL-B	remis.	PB
52	F/16	tu-media	normal	PB
53	M/28	normal	normal	PB

PIN – patient identification number, Age – in years, AML – acute myeloid leukemia, CML – chronic myeloid leukemia, ALL – acute lymphoblastic leukemia, CLL – chronic lymphocytic leukemia, HCL – hairy cell leukemia, NHL – non Hodgkin's lymphoma, tu.medias – mediastinal tumor, DN – de novo, PB – peripheral blood, BM – bone marrow.

Table 2. HLA-G specific monoclonal antibodies

Specificity	Antibody	Subclass	Provider
Intact HLA-G/β2m complex:			
	87G	IgG2a	D.E. Geraghty
	01G	IgG2a	D.E. Geraghty
	MEM-G/9	IgG1	ExBio
Free heavy chain HLA-G:			
	4H84	IgG1	M. McMaster
	MEM-G/1	IgG1	ExBio
	MEM-G/2	IgG1	V. Horejsi

tometry or resuspended for short-term cultivation in the RPMI 1640 medium supplemented with 2 mM L-glutamine, 200 μg/ml gentamicine, 0.125 μg/ml amphotericin B and 10% of heat-inactivated fetal bovine serum. When indicated, the cells were stimulated for 48 hr with 500 U/ml recombinant human IFN-γ (GIBCO BRL, USA).

Cell lines. The human choriocarcinoma cell line JEG-3 and HLA-G transfectants K562-G1, K562-G2 and LCL 721.221-G1 (.221-G1) were used as HLA-G positive controls. The transfectant .221-G1 was kindly provided by Dr. G. Frumento (National Institute for Cancer Research, Genoa, Italy). Cell lines LCL 721.221 (.221) and K562 served as HLA-G negative controls. All cell lines were propagated in the RPMI 1640 medium with 10% of heat-inactivated fetal bovine serum.

Monoclonal antibodies. Two types of HLA-G specific mAbs were used: a) mAbs directed to the intact HLA-G antigen (87G, 01G and MEM-G/9) and b) mAbs specific for the free HLA-G heavy chain (4H84, MEM-G/1 and MEM-G/2). Antibodies 87G and 01G were kindly provided by Dr. D.E. Geraghty (Fred Hutchinson Cancer Research, Seattle, USA) and mAb 4H84 was a generous gift from Dr. M.T. McMaster (University of California, San Francisco, USA). Antibodies MEM-G/9 and MEM-G/1 were purchased from Exbio (Prague, Czech Republic) and MEM-G/2 was a kind gift from Dr. V. Hořejší (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). Properties of HLA-G specific mAbs are summarized in Table 2. Antibody W6/32 recognizing native β2m-associated HLA class I antigens was obtained from Dr. J.W. Yewdell (Laboratory of Viral Diseases, NIAID, NIH, Bethesda, USA).

Acid treatment of cells. A short acid treatment of cells induces dissociation of β2-microglobulin from the HLA complex leaving the free heavy chain at the cell surface. The treatment was performed essentially according to the procedure described previously [13]. Briefly, the cell pellet was resuspended in 0.5 ml of 0.2 M citric acid-Na_2HPO_4 buffer pH 3.0 supplemented with 1% of BSA and incubated for 2 min on ice. Neutralization of samples was accomplished by excess of cold 0.1% BSA in PBS pH 7.2 and subsequent centrifugation.

Flow cytometry. To block non-specific binding of antibodies, all cell samples were first pre-incubated in 10% human AB-group serum at 4 °C for 1 hr. The cells were then washed and incubated with the first antibody for 1 hr at 4 °C. After washing, a FITC-conjugated rabbit anti-mouse IgG (Dako) was added and left to react for 30 min at 4 °C. Flow cytometric analysis was performed on a FACStar flow cytometer (Becton Dickinson, Mountain View, USA). The cells were gated using forward and side scatter. Cell viability was determined by staining with propidium iodide.

Immunoblot analysis. Cells were lysed with TENN buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Nonidet P-40) supplemented with protease inhibitors for 30 min on ice. Cell extracts were boiled in SDS-PAGE reducing sample buffer and equal amounts of total protein were separated on 10% SDS-polyacrylamide gel. Then the proteins were electroblotted onto a nitrocellulose membrane in a transfer buffer (25 mM Tris-HCl, 190 mM glycine,

20% methanol and 0.02% SDS) at 4 ˚C. The blots were blocked with 5% non-fat powder milk in PBS containing 0.01% Tween 20 and 0.01% sodium azide for 1 hr at room temperature and incubated with specific mAbs for 1 hr at room temperature. After washing the blots were incubated with peroxidase-conjugated rabbit anti-mouse IgG antibodies (Dako). Immunoreactive bands were visualized with the ECL detection system (Santa Cruz Biotechnology, Inc., USA).

RNA isolation and RT-PCR analysis. Cells (5–10x10^6) were lysed and total RNA was extracted using the Trizol reagent (Life Technologies) according to the manufacturer's instructions. Briefly, the cell lysate obtained by repeated pipetting in Trizol reagent was centrifuged at 12,000 g for 10 min. The resulting supernatant was then mixed with chloroform, the mixture was centrifuged again and subsequently total RNA was precipitated from the upper phase with isopropylalcohol. cDNA was prepared from 2 μg of total RNA by RT in a 20 μl reaction volume using the GeneAmp RNA PCR Kit (Applied Biosystems). The reaction mixture contained 1 mmole of each dNTP, 50 U of MuLV 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 99 ˚C for 5 min. The resulting cDNA was used as a template for specific amplification of HLA-G transcripts. PCR was performed in total volume of 30 μl containing 6 μl of the RT product, 0.75 U of Taq polymerase, 200 μmoles of each dNTP and 7 pmoles of each primer. The following set of primers was used: G.257F (exon 2; 5'-GGA AGA GGA GAC ACG GAA CA-3') and G.963R (exon 5; 5'-GCA GCT CCA GTG ACT ACA GC) [12]. As an internal control the β-actin gene amplification was carried out using the following primers: BGU: 5'-ATG TTT GAG ACC TTC AAC AC-3' and BGL: 5'-CAC GTC ACA CTT CAT GAT GG-3'. The PCR cycle conditions were as follows: 35 cycles at 94 ˚C/1 min, 62 ˚C/30 sec and 72 ˚C/2 min; final extension at 72 ˚C/7 min. The PCR products were analyzed by electrophoresis in 1.5% agarose gel and ehidium bromide staining.

Results

Characterization of the experimental system used for HLA-G detection. For further examination of HLA-G expression in malignant hematopoietic cells from leukemia patients we decided to expand the panel of HLA-G specific mAbs and to take advantage of mild acid treatment which allows detection of the cell surface HLA-G by flow cytometry using mAbs specific for HLA-G free heavy chain. Our experimental approach is illustrated on the mock- and pH 3.0-treated .221-G1 transfectants (Fig. 1). First the .221-G1 cells were mock- or pH 3.0-treated and subsequently incubated with HLA-G specific mAbs. Two types of antibodies were used: mAbs recognizing the native HLA-G complex (87G or MEM-G/9) and mAbs identifying the HLA-G free heavy chain (4H84 or MEM-G/1). As illustrated in the Figure 1, the HLA-G1 isoform in mock-treated cells was detected with mAbs 87G and MEM-G/9 (both recognizing the native HLA-G complex) but not with mAbs 4H84 or MEM-G/1 (detecting the HLA-G free heavy chains). The acid pretreatment of .221-G1 cells resulted in release of β2m, leaving the HLA-G1 free heavy chain on the cell surface. Therefore mAbs 4H84 or MEM-G/1 recognizing the HLA-G free heavy chains but not mAbs 87G and MEM-G/9 detecting the native HLA-G complexes were able to detect HLA-G on acid-treated .221-G1 cells (Fig. 1). These results show that both types of HLA-G specific antibodies (directed either to the intact HLA-G complexes or to the HLA-G free heavy chain) may be used in detection of the cell surface HLA-G molecules by flow cytometry. The mAb 01G recognizing the intact HLA-G complexes gave essentially the same results as mAb 87G. Similarly the results obtained with mAbs MEM-G/1 and MEM-G/2, both recognizing the HLA-G free heavy chains, were identical. Therefore the results obtained with mAbs 01G and MEM-G/2 were not included into the Tables and Figs.

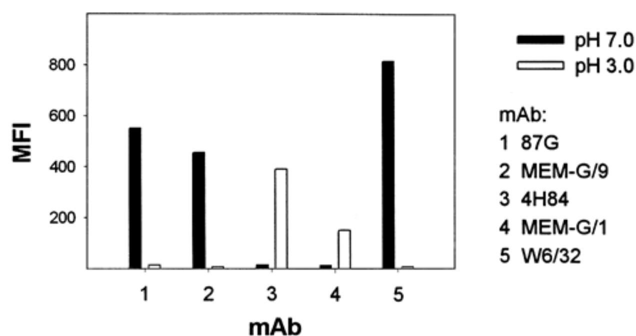
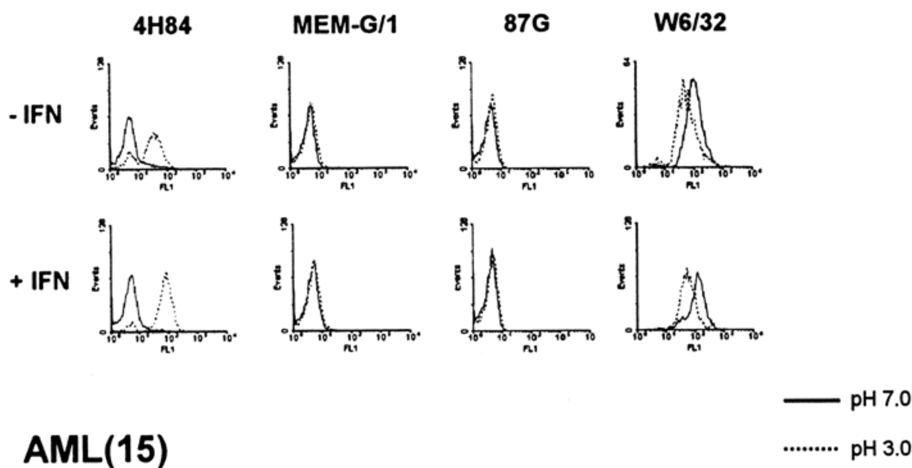


Figure 1. Binding analysis of HLA-G specific mAbs to the surface of mock- or pH 3.0-treated .221-G1 cell line. Mock- or pH 3.0-treated .221-G1 cells were stained with mAbs directed to the intact HLA-G complex (87G and MEM-G/9) or to the free HLA-G heavy chain (4H84 and MEM-G/1). The staining was evaluated by flow cytometry analysis. MFI – mean fluorescence intensity.

4H84 mAb binds to the surface of some malignant hematopoietic cells isolated from leukemia patients. To determine whether HLA-G is present on the surface of cells freshly isolated from patients characterized in Table 1 we used flow cytometry with HLA-G specific mAbs recognizing either the intact HLA-G complex or the HLA-G free heavy chains. Therefore all experiments described below include the mock- as well as pH 3.0-treated cells. In a typical experiment freshly isolated cells were first mock- or pH 3.0-treated, then incubated with 10% human serum (to block nonspecific binding of primary antibodies) and finally incubated with a particular mAb. As the IFN-γ pre-treatment may in some cells indirectly increase HLA-G expression, we



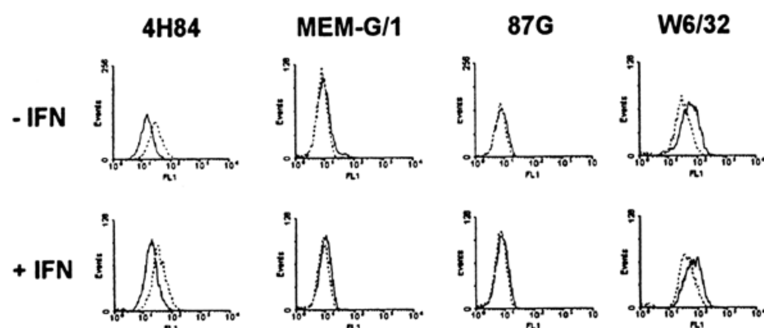


Figure 2. Binding of HLA-G specific mAbs to the surface of malignant hematopoietic cells from two patients. Leukemia cells from two patients were pre-incubated with or without IFN-γ. The cells were then mock- or pH 3.0-treated and subsequently stained with mAbs directed to the intact HLA-G complex (87G and MEM-G/9) or to the free HLA-G heavy chain (4H84 and MEM-G/1). Antibody W6/32 (anti-HLA class I) was used to monitor IFN-γ and pH 3.0 effects on expression of relevant antigens. Patient identification number is in parenthesis.

compared first the staining of cells with and without the pre-treatment. Loss or significant down-regulation of classical HLA class I antigens was not detected with the pan-HLA specific mAb W6/32 in cells of any patient. Therefore we were able to use W6/32 to monitor the IFN-γ mediated increase of HLA class I expression as well as the efficiency of the acid treatment.

Representative results are illustrated in Figures 2 and 3, respectively. Neither HLA-G specific mAbs recognizing the native HLA-G complexes nor mAbs specific to the HLA-G free heavy chains did detect HLA-G molecule on mock- treated malignant cells AML PIN 15 and ALL PIN 37 (Fig. 2) and CLL PIN 46, AML PIN 12, and ALL PIN 31 (Fig. 3). In contrast, some pH 3.0-treated patient's cells AML PIN 15, ALL PIN 37 (Fig. 2) and CLL PIN 46, AML PIN 12 (Fig. 3) were stained with mAb 4H84 recognizing the HLA-G free heavy chains, whereas the same cells were negative with other HLA-G free heavy chain recognizing mAbs namely MEM-G/1 and MEM-G/2. Other acid-treated cells, e.g. ALL PIN 31, did not bind 4H84 mAb.

As the IFN-γ pre-treatment may induce or increase binding of mAb 4H84 to acid-treated cells, all malignant cells isolated from leukemia patients summarized in Table 3 and examined for HLA-G expression were pre-treated with INF-γ. The effect of INF-γ on all the examined cells was confirmed by an increase in W6/32 binding. The INF-γ pre-incubated then mock- or acid-treated leukemia cells from any patient were not stained with mAbs 87G, MEM-G/9 or 01G identifying the native HLA-G complexes. The acid treatment enabled detection of an antigen by mAb 4H84 in cells isolated from 16 of 30 leukemia patients. The same cells, however, did not bind other mAbs (MEM-G/1 and MEM-G/2) recognizing the HLA-G free heavy chain. We have reported recently that the acid treatment induces an undesirable cross-reactivity of HLA-G specific mAb 4H84 with classical HLA class I molecules [15]. For that reason our next experiments were designed to verify whether a genuine HLA-G was expressed on some malignant cells from leukemia patients.

No antigen is detected by immunoblot analysis in malignant leukemia cells that bind mAb 4H84 after acid treatment. For immunoblot analysis, the cells freshly isolated from patients were first incubated for 48 hr with IFN-γ (500 U/ml). Then cell lysates were prepared, separated by SDS-PAGE and subsequently blotted onto nitrocellulose membranes. The blots were then incubated with the HLA-G specific mAb 4H84. Lysates of JEG-3 cells or HLA-G transfectants (221-G1, K562-G1 or K562-G2) served as HLA-G positive controls. Representative results of immunoblot analysis using mAb 4H84 are depicted in Figure 4. Expression of the HLA-G1 isoform (39 kDa) in JEG-3, .221-G1, and K562-G1 cells was clearly confirmed with mAb 4H84. The highest expression of HLA-G1 was detected in the .221-G1 transfectants. Although no binding of mAb 4H84 to acid-treated K562-G2 live transfectant cells was detected by flow cytometry (results not shown), immunoblot analysis easily detected the apparently intracellularly localized HLA-G2 isoform in this transfectant. Despite the high efficiency of

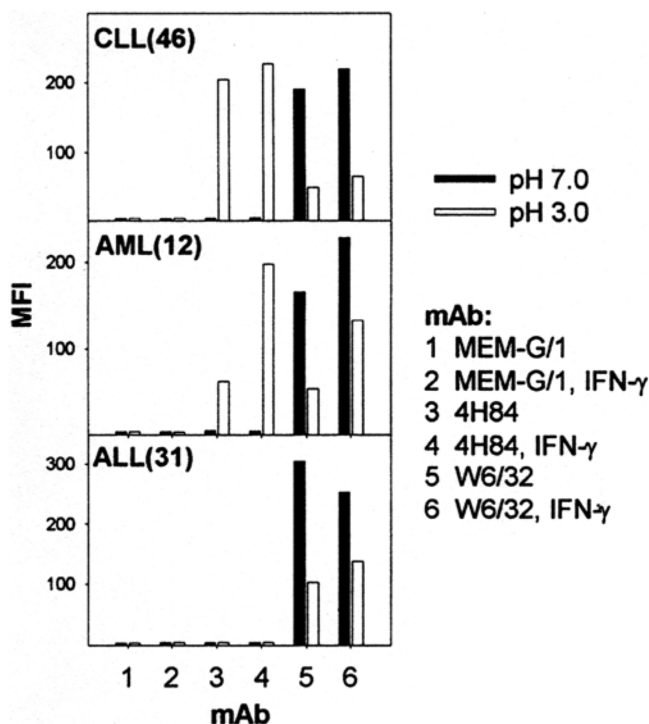


Figure 3. Three different staining patterns of leukemia cell surface using mAb 4H84. Leukemia cells from patients (pre-incubated with or without IFN-γ) were mock- or pH 3.0-treated and subsequently stained with mAbs specific to the free HLA-G heavy chain (4H84 and MEM-G/1). Antibody W6/32 (anti-HLA class I) was used to check efficacy of the IFN-γ and pH 3.0 treatment. Acid-treated leukemia cells gave with mAb 4H84 basically three different patterns of staining: 1) a high MFI even at the absence of IFN-γ (PIN 46); 2) a low MFI but significantly increased due to IFN-γ (PIN 12); 3) no staining even in the presence of IFN-γ (PIN 31). MFI = mean fluorescence intensity. Patient identification number is in parenthesis.

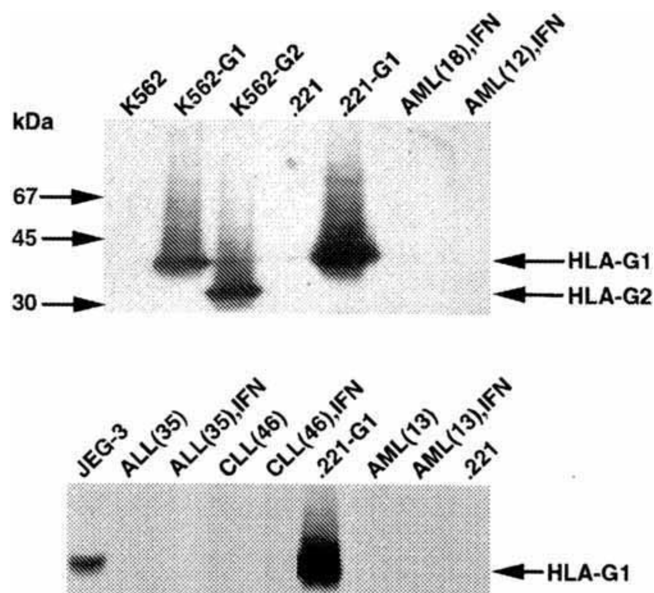


Figure 4. Immunoblot analysis of patient leukemia cells giving positive staining with mAb 4H84. Patient leukemia cells (positively stained with mAb 4H84) were analyzed using mAb 4H84. Cells .221 or K562 were served as a negative control. JEG-3 or K562-G1 cells were used as positive controls for the HLA-G1 isoform, and K562-G2 cells for HLA-G2 isoform. The relative molecular mass in kDa of reference proteins is shown on the left. Patient identification number is in parenthesis.

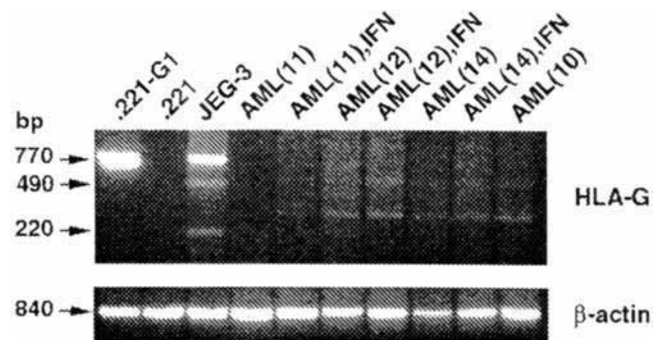


Figure 5. RT-PCR analysis of HLA-G mRNAs in patient leukemia cells giving positive staining with mAb 4H84. HLA-G specific mRNAs in patient leukemia cells were investigated. Pan-HLA-G primers (G.257F and G.963R) recognizing all alternatively spliced transripts (G1=HLA-G1 (770 bp), G2/G4=HLA-G2 or HLA-G4 (490 bp), G3=HLA-G3 (220 bp) were used. Cell lines JEG-3 and.221-G1 served as positive controls and the cell line .221 and patient leukemia cells AML (PIN 18) as negative controls. Pre-incubation of samples with IFN-γ is notified. β-actin was used as an internal control. Patient identification number is in parenthesis.

mAb 4H84 in detecting HLA-G1 and HLA-G2 isoforms in control cells, no bands were visible in the immunoblots containing leukemia lysates from cells binding mAb 4H84 (PIN 12, PIN 13, PIN 35 and PIN 46), even if corresponding cells were pre-incubated with IFN-γ (Fig. 4). Similarly, all patient samples indicated in Table 1 were found negative in immunoblot analysis with mAb 4H84.

HLA-G transcripts are not detected by RT-PCR in leukemia patient cells. The inconsistent results obtained by flow cytometry and immunoblot analysis with the mAb 4H84 prompted us to examine whether any HLA-G specific RNAs are present in patient cells that bind mAb 4H84 following the acid treatment. The RT-PCR products were obtained by amplification of total RNA prepared from patient leukemia cells and from HLA-G positive (JEG-3 and .221-G1) and negative (.221) controls. As shown in Figure 5 the specific amplification of HLA-G transcripts from JEG-3 cells revealed three bands corresponding to isoforms HLA-G1 (770 bp), HLA-G2 /HLA-G4 (490 bp) and HLA-G3 (220 bp) cDNA. The positive control .221-G1 transfectant gave one band corresponding to 770 bp. In contrast, no band was detected in .221 cells and in leukemia cells from the patient PIN 14, which did not bind mAb 4H84 after the acid treatment. No HLA-G transcripts were detected in cells from patients PIN 10, PIN 11 and PIN 12, which bound mAb 4H84 after the acid treatment, even after

Table 3. Flow cytometry analysis of surface HLA-G antigens on patient leukemia cells (pre-treated at pH 7.0 or pH 3.0)

PIN	87G		MEM-G/9		4H84		MEM-G/1		W6/32	
	pH 7.0	pH 3.0	pH 7.0	pH 3.0	pH 7.0	pH 3.0	pH 7.0	pH 3.0	pH 7.0	pH 3.0
10	–	–	NT	NT	–	+++	–	–	++++	+++/++
11	–	–	NT	NT	–	+++	–	–	++++	++
12	–	–	NT	NT	–	+/++	–	–	+++	++
13	–	–	–	–	–	+	–	–	– ++++	++
14	–	–	NT	NT	–	–	–	–	++++	+++
15	–	–	NT	NT	–	+/++	–	–	++++	+++
16	–	–	NT	NT	–	–	–	–	+++	++
17	–	–	NT	NT	–	+	–	–	+++	+++/++
18	–	–	NT	NT	–	–	–	–	++++	++
19	–	–	–	–	–	++	–	–	++++	+++/++
20	–	–	–	–	–	–	NT	NT	+++	++
21	–	–	NT	NT	–	–	NT	NT	+++	NT
27	–	–	NT	NT	–	+++	–	–	++++	++
28	–	–	–	–	–	–	–	–	+++	+++/++
29	–	–	NT	NT	–	–	–	–	++++	+++
30	–	–	–	–	–	+	–	–	+++	++/+
31	NT	NT	–	–	–	–	–	–	++++	++/+
32	–	–	NT	NT	–	–	–	–	++++	+++
33	–	–	NT	NT	–	–	–	–	++++	+
34	–	–	NT	NT	–	++	–	–	++++	+
35	–	–	NT	NT	–	+++		–	– +++	+
36	–	–	NT	NT	–	–	–	–	++++	+++
37	–	–	–	–	–	+++	–	–	++++	+++/++
38	–	–	–	–	–	–	–	–	+++	++
44	–	–	NT	NT	–	+/++	–	–	++++	+++/++
46	NT	NT	–	–	–	+++	–	–	+++	++/+
47	–	–	NT	NT	–	+	–	–	–	++++ ++
48	–	–	NT	NT	–	–	–	–	++++	+
49	–	–	NT	NT	–	–	NT	NT	+++	++/+
50	NT	NT	–	–	–	++	NT	NT	+++	++
52	–	–	NT	NT	–	–	–	–	++++	+++
53	–	–	NT	NT	–	–	–	–	++++	++

PIN – patient identification number, NT – not tested, HLA-G specific mAbs: 87G, MEM-G/9, 4H84, MEM-G/1

pre-incubation with IFN-γ. RT-PCR analysis performed at the highest possible sensitivity revealed slight bands in patient samples, nevertheless their positions did not correspond to any of the HLA-G isoforms. All patient samples indicated in Table 3 as positive for mAb 4H84 binding due to the acid treatment were found negative for the presence of HLA-G specific mRNAs.

Discussion

Currently the prevailing concept is that HLA-G is responsible for protection of the fetal semiallograft from an attack by maternal NK cells and that some tumor cells also express HLA-G to achieve the same protective effect [2, 5, 9, 17]. Although the HLA-G transcriptional activity has been observed in many tumor cell lines and tumor tissues obtained from patients, the HLA-G protein has been found only in very few samples [6, 7, 10, 11, 18]. Therefore a role of HLA-G in protecting tumor cells has yet to be elucidated.

We focused our studies to hematologic malignances because they have not yet been extensively analyzed for expression of the HLA-G molecules. The expression of HLA-G transcripts but not HLA-G protein has been reported in a number of leukemia cell lines and leukemia cells from patients [1, 2, 17, 18]. In our previous studies, we have also failed to detect HLA-G antigen in established tumor cell lines of different origin as well as in malignant hematopoietic cells of leukemia patients [14, 16]. In contrast, others have detected HLA-G in 6 of 28 patients with AML, but only after the IFN-γ stimulation [8]. In that study, however, a non-specific binding of HLA-G specific mAb 87G to FcγRI, which also is upregulated by IFN-γ was not conclusively excluded.

In order to elucidate such conflicting findings, we extended analysis of the HLA-G expression in malignant hematopoietic cells isolated from leukemia patients. The HLA-G expression in cells from leukemia patients was examined with an expanded panel of HLA-G specific mAbs. The flow cytometry analysis included the pH 3.0-treated

and untreated cells. Such experimental conditions allowed detection of the surface HLA-G by flow cytometry not only with mAbs recognizing the intact HLA-G complexes (87G, 01G and MEM-G/9) but also with mAbs detecting the HLA-G free heavy chain on acid-treated cells (4H84, MEM-G/1 and MEM-G/2) [15]. All HLA-G specific mAbs with the exception of mAb 4H84, did not bind to the cells from any leukemia patient even after the IFN-γ treatment. The results obtained with mAb 4H84 recognizing all HLA-G isoforms are of particular interest. We found that acid-treated cells isolated from 14 of 30 analyzed leukemia patients bound mAb 4H84. The pre-treatment in most cases increased this binding, and in some cases the binding of mAb 4H84 was detected only after the IFN-γ treatment. Recently we demonstrated that mAb 4H84 recognized on acid-treated cells not only the non-classical HLA-G but also the classical HLA class I antigens. Therefore the positive staining of some acid-treated cells from leukemia patients did not provide sufficient evidence for the presence of HLA-G. To distinguish the two types of HLA antigen we performed immunoblot analysis. In such analysis mAb 4H84 detected HLA-G but not classical HLA class I molecules [15]. The immunoblot analysis did not reveal the presence of HLA-G in any patient cells binding mAb 4H84 after the acid treatment. Staining with mAb W6/32 confirmed that classical HLA class I antigens were present in cells from all patients. Loss or significant down-regulation of classical HLA class I antigens was not detected in any of the patient cells using W6/32. Therefore we conclude that mAb 4H84 recognized classical HLA class I antigens on patient cells that bound mAb 4H84 following the acid treatment. This conclusion is further supported by the inability of RT-PCR to detect HLA-G mRNAs in any cell from all leukemia patients binding 4H84 after the acid treatment. Loss or significant down-regulation of classical HLA class I antigens was not detected in leukemia cells from any patient. This is in agreement with infrequent loss or downregulation of HLA class I antigens in leukemia [4]. In summary our results provide further evidence that the non-classical class I molecule, HLA-G does not play a significant role in protection of malignant hematopoietic cells from an immune attack.

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