

Functional Role of Human Leukocyte Antigen-G Up-Regulation in Renal Cell Carcinoma¹

Jürgen Bukur, Vera Rebmann, Hans Grosse-Wilde, Hans Luboldt, Herbert Ruebben, Ingo Drexler, Gerd Sutter, Christoph Huber, and Barbara Seliger²

Third Department of Internal Medicine, Johannes Gutenberg University, 55101 Mainz [J. B., C. H., B. S.]; Institute of Immunology, University Hospital of Essen, 43147 Essen [V. R., H. G.-W.]; Department of Urology, University Hospital of Essen, 43147 Essen [H. L., H. R.]; Technical University, Institute of Virology, 81675 Munich [I. D.]; and Gesellschaft für Strahlenschutz-forschung Research Center, Institute of Molecular Virology, 81675 Munich [G. S.], Germany

ABSTRACT

The nonclassical HLA-G molecule exhibits a limited tissue distribution and exerts multiple immune regulatory functions. Recent studies indicate that HLA-G expression plays a key role in the induction of immune tolerance and may represent a novel immune escape mechanism of tumor cells. Despite a high frequency of tumor-infiltrating T lymphocytes in renal cell carcinoma (RCC) lesions, outgrowth of tumor cells occurs that might be attributable to abrogation-efficient antitumor responses. To delineate the potential role of HLA-G in RCC immunology, the HLA-G expression pattern and its functional consequences on immune responses were analyzed in cell lines and lesions derived from primary RCC lesions. A heterogeneous constitutive and IFN- γ -inducible HLA-G mRNA and protein expression was found in 12.5% of RCC cell lines but not in autologous normal kidney cells. Western blot analysis of 37 primary RCC lesions revealed HLA-G protein expression in 27% of RCC lesions. Functional studies performed with alloreactive natural and lymphokine-activated killer cells as well as antigen-specific CD8⁺ T-cell populations demonstrated that HLA-G expression inhibits lysis of RCC cells by these different immune effector cells, whereas HLA-G⁻ normal kidney cells were recognized. Furthermore, the HLA-G-mediated counteraction of immune response could be restored by antibody blocking experiments. Thus, aberrant HLA-G expression is found at a relatively high frequency in RCC and might participate in evasion of these tumor cells from immunosurveillance.

INTRODUCTION

HLA class I abnormalities, which are often attributable to defects in the MHC class I antigen processing pathway, have a decisive influence on the metastatic capacity of tumors and the host immunosurveillance, thereby resulting in resistance of tumor cells to CTL-mediated lysis and susceptibility to NK³ cell-mediated killing (1). Up-regulation of the nonclassical HLA class I molecule HLA-G can also modulate immune responses by protecting HLA class I-deficient targets from NK cell-mediated lysis. In contrast to the classical HLA class I antigens, HLA-G is characterized by a limited polymorphism and a tissue-restricted, selective expression, mainly at the materno-fetal interface and the thymus. A major feature of HLA-G is the alternative splicing of its primary transcript creating at least seven different isoforms, four membrane-bound (HLA-G1 to HLA-G4), and three soluble proteins (sHLA-G: HLA-G5, HLA-G6, and HLA-G7; Refs. 2, 3).

Data regarding HLA-G expression in tumors are highly conflicting.

Although an up-regulation of HLA-G expression has been reported in different solid tumors, controversial information exists about its clinical and functional relevance (4–6). The frequency of HLA-G expression significantly varied between the different tumor types analyzed but also between the same tumor entity depending on the study (4–9). HLA-G mRNA and/or protein expression was demonstrated in T- and B-cell lymphomas, in lung carcinoma, sarcoma, bladder cancer, glioblastoma, breast carcinoma, colorectal cancer, melanoma, as well as RCC (4, 10–13), but the number of tumors analyzed is still limited.

Experimental evidence of HLA-G-mediated suppression of T-cell and NK cell responses was obtained from *in vitro* studies (4, 14). HLA-G expression on tumor cells in association with their ability to interact with the immunoglobulin-like KIRs of NK and T cells suggest that HLA-G molecules protect tumor cells from its host immune attack. To additionally evaluate the frequency and biological relevance of HLA-G expression in tumors, both the constitutive and/or IFN- γ -regulated expression was monitored in cell lines and tissue samples derived from RCC lesions and autologous normal kidney epithelium. In addition, the role of HLA-G expression in the control of immune responses directed against RCC was investigated. Our data propose that HLA-G expression in RCC cells negatively interferes with the host immune response by inhibiting CTL and LAK and NK cell function.

MATERIALS AND METHODS

Cell Lines and IFN- γ Treatment. The cell line MZ2733RC and the autologous normal kidney cell line MZ2733NN were recently established from a patient with metastatic RCC (J. B. and B. S., personal communication). The other RCC cell lines used in this study have been described elsewhere and were kindly provided by Peter I. Schrier (University of Leiden, Leiden, the Netherlands) and Alexander Knuth (Nordwest Krankenhaus, Frankfurt, Germany; Ref. 15). The HLA-G⁺ human choriocarcinoma cell line JEG3, the HLA-A2⁻, HLA-G⁻ normal kidney cell line MZ2789NN (J. B. and B. S., personal communication), and the HLA class I⁻, LAK cell-sensitive erythroleukemic cell line K562, served as controls in functional assays. The HLA-A2-restricted tyr-specific CTL and the HLA-A2⁺, tyr-expressing NA8 melanoma cells have been described elsewhere (16). All cell lines were maintained in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine, penicillin, and streptomycin. IFN- γ treatment was performed for 48 h using 20 ng/ml recombinant human IFN- γ (Thomae, Biberach, Germany) as described recently (15).

Tumor Specimen and Patients' Characteristics. Tissue specimens from tumor and autologous adjacent normal kidney tissue were obtained from 37 patients who had undergone radical nephrectomy. All tumor specimens were classified as clear cell adenocarcinoma. Stage of disease, tumor invasion, and lymph node involvement according to the tumor-node-metastasis classification system, as well as patients' age and sex are summarized in Table 1.

mAbs and Flow Cytometry. The following mAbs were used: the mAb w6/32 recognizing a monomorphic epitope of HLA class I in association with β_2 -microglobulin (17); the anti-HLA-A2 mAbs mA2.1 and BB7.2 (16); the anti-HLA-G-specific mAbs MEM-G/9 (IgG1) and MEM-G/1 (IgG1) both purchased from Exbio (Prag, Czech Republic; Refs. 18, 19); and the isotypic murine control antibodies IgG1, IgG2a, and the FITC-conjugated goat-anti-mouse immunoglobulin as secondary antibody, all purchased from Beckman

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² To whom requests for reprints should be addressed, at Johannes Gutenberg-University, Third Department of Internal Medicine, Langenbeckstr. 1, 55101 Mainz, Germany. Phone: 06131-17-6760; Fax: 06131-17-5635; E-mail: B.Seliger@3-med.klinik.uni-mainz.de.

³ The abbreviations used are: NK, natural killer; HC, heavy chain; KIR, killer-inhibitory receptor; LAK, lymphokine-activated killer; mAb, monoclonal antibody; MVA, modified vaccinia virus Ankara; PBMC, peripheral blood mononuclear cell; RCC, renal cell carcinoma; sHLA, soluble HLA; tyr, tyrosinase.

Table 1 Characteristics of patients and RCC lesions

ID no.	Age (yr)	Sex	Tumor staging			HLA-G expression		
			T	N	M	Grading	Normal	Tumor
1	70	M	2	0	0	I	-	-
2	62	M	2	0	0	I	-	+
3	51	M	1	0	0	n.a.	n.a.	-
4	44	M	2	0	0	II	n.a.	-
5	71	M	2	0	0	I	-	-
6	59	F	3	0	0	III	-	-
7	43	F	2	0	0	II	-	-
8	66	F	3	0	1	II	-	-
9	40	M	3	0	x	II	n.a.	+
10	73	F	2	0	0	I	-	+
11	71	M	3	0	0	I-II	-	-
12	58	M	4	2	x	III	n.a.	-
13	58	M	4	2	x	III	-	+
14	40	M	3	0	0	I-II	-	+
15	59	M	2	0	0	III	-	-
16	74	M	3b	0	0	II	n.a.	-
17	54	M	2	0	0	III	n.a.	-
18	71	F	1	0	0	III	n.a.	-
19	73	M	2	0	0	III	n.a.	+
20	64	F	2	0	0	III	n.a.	+
21	40	M	2	0	0I	n.a.	n.a.	-
22	77	F	3	0	0	III	-	-
23	62	F	1	0	0	n.a.	-	-
24	67	F	3a	0	0	II	n.a.	+
25	67	M	2	0	0	n.a.	n.a.	-
26	73	M	2	0	0	III	-	-
27	72	F	2	0	0	III	-	+
28	64	M	2	0	0	II	-	-
29	68	M	1	0	0	I	-	-
30	46	F	1	0	0	I	-	-
31	49	M	2	0	0	n.a.	n.a.	+
32	56	F	3	0	0	III	-	-
33	55	M	2	0	0	II	-	-
34	56	M	2	0	0	I	-	-
35	53	M	2	0	0	I	-	-
36	69	M	2	0	0	II	-	-
37	52	M	2	X	0	I	-	-

^a Thirty-seven randomly selected lesions of RCC patients were analyzed for HLA-G protein expression by immunoprecipitation, SDS-PAGE, and Western blotting using the HLA-G-specific mAbs MEM/G9 and MEM/G1 as immunoprecipitation or MEM/G1 as detection antibodies after Western blotting. n.a., not available; -, no HLA-G expression detectable; +, HLA-G expression detectable.

Coulter (Krefeld, Germany). The alkaline phosphatase-labeled goat antimouse IgG antibody was obtained from Dianova (Hamburg, Germany).

For flow cytometry, cells were incubated with the respective primary mAbs or isotype-matched control antibody for 30 min on ice followed by an additional 30-min incubation with the secondary antibody. After washing in PBS/1% heat-inactivated FCS, cells were analyzed on a flow cytometer (Coulter Epics XL MCL; Beckman Coulter).

Reverse Transcriptase-PCR Analysis and Southern Blotting. HLA-G-specific amplifications were performed as described by Real *et al.* (6) with minor modifications using the HLA-G-specific primer pairs that coamplify the HLA-G isoforms HLA-G1, HLA-G2, HLA-G3, HLA-G4, HLA-G5, and HLA-G6: G.257 (sense: 5'-GGAAGAGGAGACACGGAACA-3') and G.1225 (antisense: 5'-TGAGACAGAGACGGAGACAT-3') and the β -actin-specific primers (sense: 5'-GAAGCATTGCGGTGGACGAT-3'; antisense: 5'-TCCTGTGGCATCCACGAAACT-3'). Amplification products were size fractionated on 1% ethidium bromide containing agarose gels. Gels were blotted onto nitrocellulose membranes (Roche Diagnostics), which were hybridized with a biotin-labeled exon 5-specific HLA-G oligonucleotide and additionally processed as described recently (6).

Immunoprecipitation, SDS-PAGE, and Western Blotting. Ten μ g of mAb MEM-G/9 were conjugated to a 1-ml suspension of 1×10^8 immunomagnetic beads (Dynabeads M280; Dynal, Hamburg, Germany) by an overnight incubation at 4°C. After intensive washings, the MEM-G/9-precoated immunobeads were filled up to 1 ml and stored until usage. SDS-PAGE and Western blotting were performed as described previously (20). The precipitation of HLA-G and sHLA-G molecules was performed either with 1-ml cell lysate of 5×10^6 cells or 1-ml cell lysate obtained from 60- μ g tissue sample (21) or 6 ml of spent medium from the respective cell lines incubated overnight with 250 μ l of immunomagnetic beads precoated with the mAb MEM-G/9.

Upon elution, antigens were subjected to SDS-PAGE and subsequently analyzed by Western blotting using mAb MEM-G/1 for the detection of HLA-G and sHLA-G molecules, which were visualized by a chemiluminescent detection kit (Perbio Science, Bonn, Germany).

Generation of LAK Cells and NK Cells. LAK cells were generated from freshly isolated or thawed PBMCs obtained from buffy coats of HLA-A1- and HLA-A2-matched healthy donors. PBMCs were separated by Ficoll gradient (Pharmacia, Uppsala, Sweden), washed, resuspended in complete RPMI supplemented with 10% human AB serum, and 2 ml (1×10^6 cells/ml) were plated into 24-well tissue plates in presence of 6000 units/ml recombinant human interleukin-2 (Proleukin; Roche Diagnostics, Basel, Switzerland) and incubated for 3 days at 37°C, 5% CO₂.

NK cells were isolated from 3×10^7 PBMCs resuspended in 240 μ l of PBS supplemented with 0.5% BSA and 2 mM EDTA by negative selection with hapten-conjugated antibodies directed against CD3, CD14, CD19, CD36, and anti-IgE using an antihapten antibody coupled to microbeads as a secondary antibody according to the manufacturers protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). NK cells were cultured overnight in RPMI 1640 supplemented with 15% FCS, 2 mM glutamine, 1 mM sodium-pyruvate, and 1×10^6 nonessential amino acids (Invitrogen, Krefeld, Germany) in the presence of 500 units/ml recombinant human interleukin-2 before NK cells were used for cytotoxicity experiments. The purity of isolated subpopulations used in the experiments was >90% as determined by flow cytometry.

Cytotoxicity Assays. Cytotoxicity assays were performed using 5×10^5 – 1×10^6 target cells labeled with 50–100 μ Ci ⁵¹sodium chromate for 1 h at 37°C, 5% CO₂. For blocking experiments, RCC and normal kidney epithelium cells were preincubated with the anti-HLA class I-specific mAb w6/32 or the corresponding anti-immunoglobulin isotype control. A total of 1×10^3 of ⁵¹Cr-labeled cells was incubated with the respective effectors at different E:T ratios for 4–6 h at 37°C. Specific lysis was calculated as [cpm (effector cells) – cpm (spontaneous)]/cpm (Triton X-100) – cpm (spontaneous)] $\times 100\%$. The cytolytic activity of HLA-A2-restricted tyr-specific T cells was assessed on MVA-tyr-infected targets using the standard chromium release assay as described recently (22).

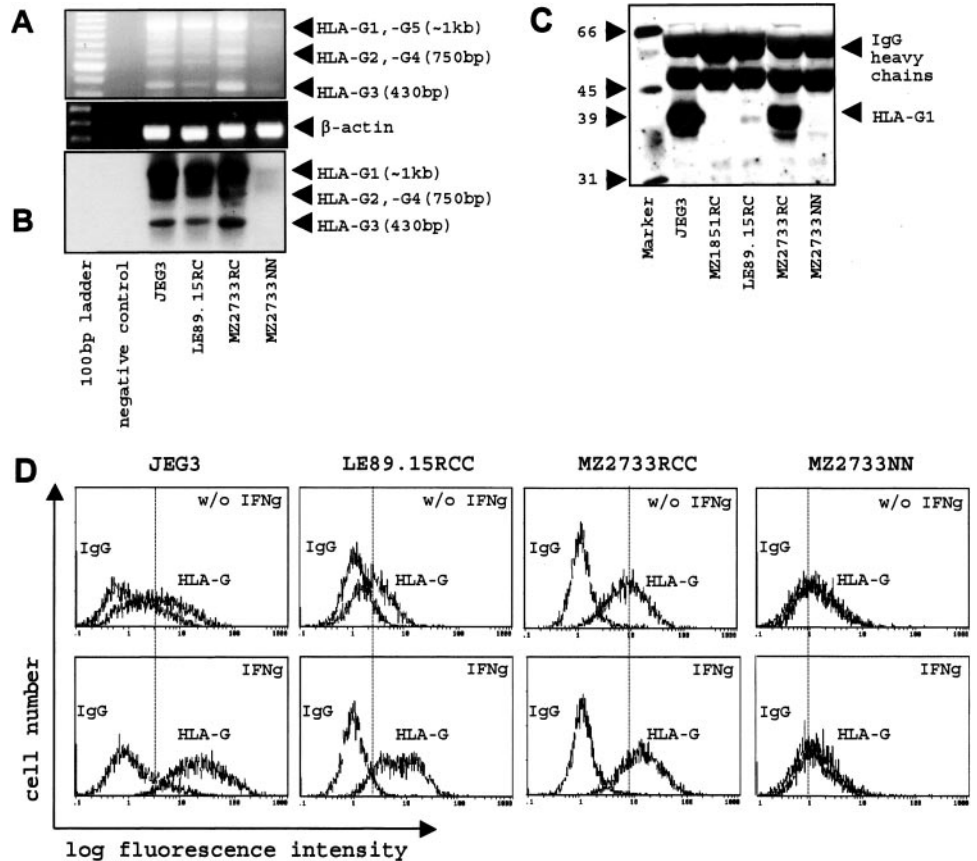
Statistics. All data are representative experiments performed at least twice. Differences between two groups were analyzed for significance by Wilcoxon's test for paired samples ($P < 0.05$).

RESULTS

Expression of Multiple HLA-G mRNA Transcripts in RCC Cell Lines. To address the biological significance of HLA-G expression in human RCC, HLA-G expression was studied in 24 RCC cell lines and 8 autologous normal kidney cells by reverse transcriptase-PCR analysis using pan-HLA-G-specific primers (6). The HLA-G⁺ choriocarcinoma cell line JEG3 served as control. In total, 12.5% of RCC cell lines demonstrated HLA-G-specific transcripts, whereas normal kidney cells lack HLA-G expression. As representatively shown in Fig. 1A, alternative splicing patterns of HLA-G were detected in the RCC cell lines MZ2733RC and LE89.15RC and JEG3 control cells but not in autologous kidney cells. However, the transcription level of the different HLA-G isoforms varied between the HLA-G⁺ RCC cell lines. The constitutive HLA-G transcription was additionally confirmed by Southern blot analysis using an exon 5-specific probe (Fig. 1B).

Constitutive and IFN- γ -inducible HLA-G Expression in RCC Cell Lines but not in Normal Kidney Cells. To verify the translation of HLA-G transcripts into HLA-G protein, flow cytometric analyses were performed using the HLA-G-specific mAb MEM-G/9, as well as the anti-HLA class I mAb w6/32. All HLA-G mRNA-expressing RCC cell lines demonstrated a significant HLA-G surface expression. As representatively shown for the RCC cell lines LE89.15RC and MZ2733RC, the constitutive HLA-G surface expression was at least comparable with that of HLA-G⁺ JEG3 control cells, whereas the corresponding normal kidney cells MZ2733NN failed to express HLA-G protein (Fig. 1D). IFN- γ treatment caused on up-

Fig. 1. Detection of HLA-G-specific mRNA isoforms and proteins in different RCC cell lines. A-C, 100 ng of DNase I-digested total cellular RNA from different RCC cell lines and corresponding normal kidney cells were used for analysis of HLA-G and β -actin expression. HLA-G transcripts were detected in the positive control JEG3 as well as the RCC cell lines LE89.15RC and MZ2733RC but not detected in the normal kidney cells MZ2733NN. A, one percent TAE-agarose gels of size-separated HLA-G amplicons detecting HLA-G1 (980 bp), HLA-G2, HLA-G4 (750 bp), and HLA-G3 (430 bp) and β -actin amplicons. B, Southern blot confirming the specificity of HLA-G amplicons. C, detection of HLA-G1 molecules in RCC cell lines using the mAb MEM-G/9. A HLA-G1-specific protein pattern with a typical molecular size of M_r 39,000 was obtained from TX114 detergent lysate of the HLA-G-positive cell line JEG3 and RCC cell line LE89.15RC and MZ2733RC, but not by the autologous kidney cells MZ2733NN and the RCC cell line MZ1851RC. D, constitutive and IFN- γ -mediated HLA-G1 expression in RCC cell lines. The untreated and IFN- γ -treated RCC cell lines LE89.15RC and MZ2733RC, the normal kidney cell line MZ2733NN as well as the choriocarcinoma cell line JEG3 were subjected to flow cytometry as described in "Materials and Methods" using the anti-HLA-G1-specific mAb MEM-G/9 and the murine isotypic control antibody. Constitutive and IFN- γ -mediated HLA-G expression was detected in the cell lines JEG3, LE89.15RC, and MZ2733RC, but not in the normal kidney cells MZ2733NN.



regulation of HLA-G surface expression in HLA-G⁺ cell lines but not in HLA-G⁻ normal kidney cells. It is noteworthy that all RCC cell lines and normal kidney cells expressed high levels of the classical HLA class I antigens, which were additionally enhanced by IFN- γ stimulation (data not shown).

Detection of Membrane-anchored and Soluble HLA-G Molecules in RCC. To confirm the flow cytometric data, membrane-bound and soluble HLA-Gs were immunoprecipitated from cell lysate or culture supernatant of HLA-G⁺ RCC and HLA-G⁻ normal kidney cells using the mAb MEM-G/9 as capture and mAb MEM-G/1 as detection reagent. HLA-G⁺ JEG3 and HLA-G⁻ MZ1851RC cells served as positive and negative controls, respectively. Using the mAb MEM-G/9, HLA-G1 molecules with a molecular size of M_r 39,000 were immunoprecipitated from the HLA-G⁺ JEG3, MZ2733RC, and LE89.15RC cell lines, but not from MZ1851RC and the normal kidney cell line MZ2733NN (Fig. 1D). In addition, sHLA-G1 molecules with the typical molecular weight of M_r 35,000 were detected in the supernatants of the RCC cell lines MZ2733RC and LE89.15RC (data not shown).

HLA-G Protein Expression in RCC Lesions *in Vivo*. Western blot analysis was performed to determine HLA-G protein expression in a series of primary RCC lesions and autologous normal kidney tissues. Ten of 37 RCC samples expressed high levels of HLA-G protein, suggesting that HLA-G expression frequently occurs in RCC, whereas normal kidney cells lack HLA-G expression (Fig. 2). No correlation was found between HLA-G expression and clinical parameters such as tumor staging, grading, and age of the patients.

Effect of HLA-G Expression on Immune Response. To elucidate the functional relevance of constitutive HLA-G expression in RCC cells, different immune effector cells were used for cytotoxicity assays. For the determination of the CTL-mediated cytotoxicity, unin-

ected and MVA-tyr-infected HLA-A2⁺, HLA-G⁺ RCC cell line MZ2733RC and its autologous HLA-G⁻ normal kidney counterpart MZ2733NN were used as targets for HLA-A2-restricted, tyr-specific CTL. The HLA-A2⁺, HLA-G⁻ melanoma cell line NA8 and the HLA-A2⁻, HLA-G⁻ normal kidney cell line MZ2789NN served as controls. NA8 and HLA-A2⁺, MVA-tyr-infected MZ2733NN cells were efficiently recognized by the HLA-A2-restricted tyr-specific CTL, whereas all tyr⁻ cell lines as well as the HLA-A2⁻, MVA-tyr⁺ normal kidney cells MZ2789NN were not recognized. The relevance of constitutive HLA-G molecules for protection of RCC from CTL-specific lysis was demonstrated because the CTL-mediated lysis of MVA-tyr⁺ HLA-G⁺ MZ2733RC cells was significantly down-regulated when compared with the MVA-tyr-infected autologous

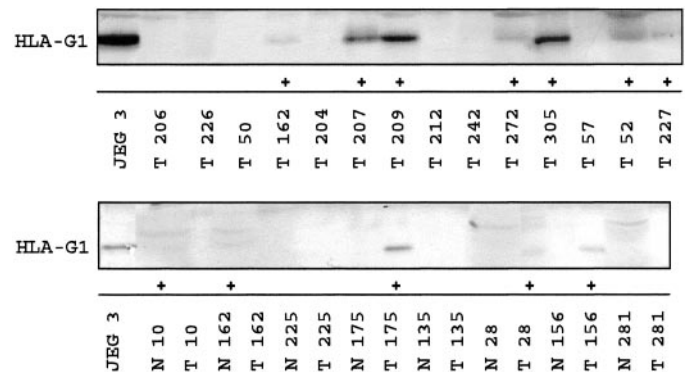


Fig. 2. Analysis of HLA-G1 protein expression in RCC lesions. Proteins from RCC lesions and corresponding normal kidney tissues were assessed by Western blot analysis as described in "Materials and Methods." The cell line JEG3 served as positive control. T and N indicate tumor lesion and autologous normal kidney specimen, respectively.

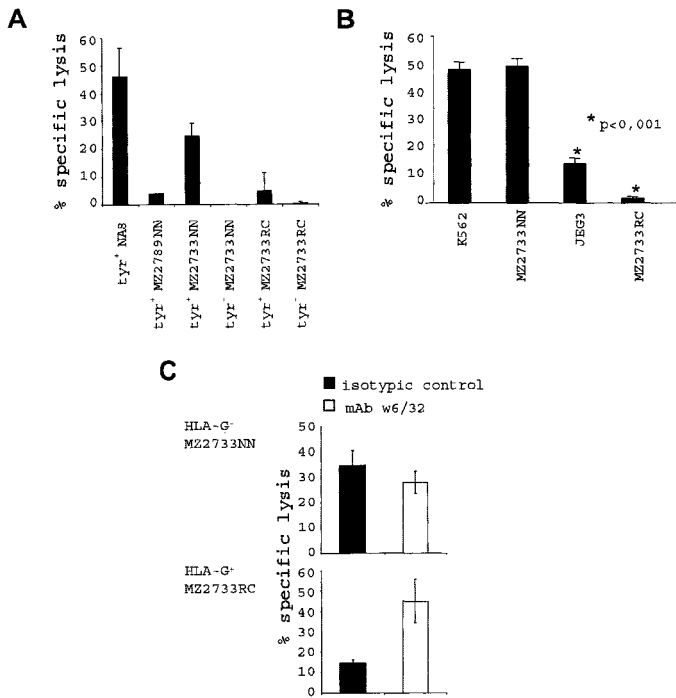


Fig. 3. Impaired CTL- and LAK-mediated cytotoxicity of HLA-G-expressing tumor cells. **A**, tyr-directed lysis of different cell lines after infection with recombinant modified MVA-tyr. Uninfected and MVA-tyr-infected cells were incubated with HLA-A2-restricted tyr-specific CTL at different E:T ratios (30:1, 10:1, 3:1, and 1:1). Three independent experiments were performed and the results are expressed as percentage of specific lysis \pm SD. Representative data using an E:T ratio of 30:1 are shown. The HLA-A2⁺, tyr⁺ NA8 melanoma cells served as positive, the MVA-tyr-infected HLA-A2⁻ MZ2789NN cells as negative control, respectively. Both NA8 cells (46 \pm SD10%) and the HLA-A2⁺ tyr⁺ MZ2733NN (24 \pm SD5%) but not the uninfected MZ2733NN (0%) and MZ2733RC (0%) cells were recognized by the HLA-A2-restricted, tyr-specific CTL. CTL-mediated lysis of HLA-A2⁺, tyr⁺ MZ2733RC (5 \pm SD7%) cells was strongly down-regulated. **B**, differential LAK cell-mediated recognition of normal kidney cells and RCC cells. The generation of LAK cells and the cytotoxicity assays were performed as described in "Materials and Methods." LAK cells from 10 HLA-A1, HLA-A2-matched healthy donors recognized the target cells at the indicated E:T ratio (30:1) in a chromium release assay. The K562 and MZ2733NN were LAK cell-sensitive, whereas the lysis of JEG3 as well as MZ2733RC cells was significantly reduced (Wilcoxon's test was performed; $P < 0.001$). The results are expressed as mean percentage lysis using 10 independent LAK cell populations. **C**, restoration of cytotoxicity by antibody blockade. Targets cells were preincubated with saturating amounts of w6/32 (□) or isotope control antibodies (■) for 30 min before LAK cells were added as effectors at an E:T ratio of 35:1, and the results are expressed as percentages of specific lysis \pm SD obtained in 6-h ⁵¹Cr release assays. Three independent experiments were performed, and the lysis of HLA-G⁻ MZ2733NN normal kidney cells (35 \pm SD6%) was reduced in presence of w6/32 (28 \pm SD4%), whereas the lysis of HLA-G⁺ MZ2733RC tumor cells (15 \pm SD2%) was 3-fold enhanced (45 \pm SD11%).

HLA-G⁻ kidney cells (Fig. 3A). The impaired susceptibility of MVA-tyr⁺ MZ2733RC cells was not attributable to reduced HLA-A2 surface antigen expression because both MZ2733NN and MZ2733RC cells expressed similar HLA-A2 surface levels (data not shown). In this context, it is noteworthy that all RCC cells and normal kidney cells express the same levels of HLA-E (data not shown). Thus, HLA-G expression appears to participate in the functional inhibitory effect on CTL-mediated lysis of the HLA-G⁺ RCC cell lines.

To investigate whether the HLA-G expression on RCC also affects LAK cell function, LAK cells generated from PBMCs of 10 healthy donors were used as effectors in cytotoxicity assays. JEG3 and K562 cells served as controls, respectively. All LAK cells efficiently recognize the HLA-G⁻ normal kidney cells MZ2733NN and K562 cells with lysis rates ranging between 40 and 55%. In contrast, the lysis of HLA-G⁺ JEG3 and MZ2733RC cells was significantly reduced ($P < 0.001$) with values ranging between 12 and 2%, respectively (Fig. 3B). Blocking experiments with the pan anti-HLA class I mAb w6/32, which reacts with HLA-G and the negative immune regulatory

molecule HLA-E, were performed to restore LAK activity. As shown in Fig. 3C, saturating amounts of mAb w6/32 enhanced LAK-specific killing of HLA-G⁺ RCC cells, but had no effect on the autologous kidney cells.

To additionally elucidate the effector populations inhibited by HLA-G expression of RCC cells, LAK and NK cells generated from the same healthy volunteers were used for cytotoxicity assays. As expected, HLA-G⁺ MZ2733RC cells were resistant to both LAK and NK cell-mediated lysis, whereas the HLA-G⁻ MZ2733NN and K562 control cells were not protected from lysis (data not shown). However, there exist quantitative differences between the level of protection using LAK and NK cells.

DISCUSSION

The aim of our study was to elucidate the biochemical and functional properties of HLA-G expression on RCC cells and its role as an immune escape mechanism of this tumor entity. Using reverse transcriptase-PCR analysis, the alternative splicing variants of HLA-G were detected in 12.5% of RCC cell lines analyzed but not in normal kidney cells. The corresponding membrane-bound HLA-G1 isoform could also be detected at the protein level (Figs. 1C and 2). The correlation between alternative transcript levels and protein expression in RCC cell lines argues against a posttranscriptional control of HLA-G expression in RCC, which has been demonstrated in other tumor lesions. In addition, HLA-G protein expression was detected in 27% of RCC lesions. Although the literature about HLA-G expression on tumors is conflicting (4), our data confirm and extend a previous report that provided the first evidence of HLA-G expression in RCC (10). However, the frequency of HLA-G expression between RCC cell lines and tumor lesions differed. The incidence of HLA-G expression is probably higher *in vivo* than in *in vitro* RCC cell cultures as it has been previously reported also for melanoma (8).

The hypothesis of HLA-G expression as a possible tumor immune escape mechanism was strengthened by recent data demonstrating that HLA-G surface expression in melanoma and glioblastoma cells exerts significant immune inhibitory function on different immune effector cells (4, 13, 23). Our data suggest for the first time that HLA-G-expressing RCC cell lines were less sensitive to CTL, LAK, and NK cell-mediated cytotoxicity than HLA-G⁻ normal kidney cells. However, evidence linking inhibition of cytotoxicity with HLA-G expression is still correlative. It can be speculated that the inhibition of LAK activity might result from interactions between HLA-G and the KIRs present on activated effector cells. These results are in line with experiments of Stanley *et al.* (24), demonstrating resistance of RCC to NK cell lysis via the CD94 receptor. It is generally accepted that the level of HLA class I surface antigens on tumors is inversely associated with their resistance to NK and/or LAK cell-mediated lysis. However, the differential recognition of the MZ2733 system by allogeneic, innate effector cells appears to be independent of the HLA class I expression level because both MZ2733RC and MZ2733NN cells express same amounts of HLA class I surface molecules, suggesting an immunoregulatory role of HLA-G coexpressed in presence of HLA class I antigens. An indirect up-regulation of other inhibitory molecules such as HLA-E can be excluded. This is based on the fact that HLA-E expression was found on RCC and normal kidney cells (data not shown). If HLA-E negatively interferes with the immune response an inhibitory effect would have been noticed in HLA-G⁻ cells (2, 4).

Because KIRs are also present on T lymphocytes, HLA-G molecules may also affect T-cell function, thereby enlarging their capacity to block both NK and T-cell responses (14). Indeed, a decreased CTL-mediated recognition of HLA-G⁺ MZ2733RC cells in comparison to its autologous HLA-G⁻ normal kidney cells was observed

using tyr as a surrogate antigen. Thus far, limited information exists about the role of sHLA-G in immune response, sHLA-G has recently been demonstrated to prevent T-cell- and NK cell-mediated cytotoxicity (25). However, one might speculate that the CTL and NK cell-based immune responses might be additionally impaired by sHLA-G released from RCC cells. *In vivo* HLA-G1 protein expression was demonstrated in ~27% of RCC specimens, which additionally corroborate the functional data obtained *in vitro* in RCC cell lines. HLA-G expression has been demonstrated in tumors of distinctive histology, but its overall relevance as an alternative principle of immune escape is still controversially discussed (4). This is further complicated by the limited availability of HLA-G-specific mAb, which could be routinely used for immunohistochemical staining of paraffin-embedded tissue sections (18, 19). In this context, it is noteworthy that the commercially available antibody BFL1 lack HLA-G-specific reactivity as demonstrated by the HLA-G workshop in 2000 (19).

Thus, HLA-G expression on RCC cells *in vitro* and *in vivo* can lead to impaired immune recognition by CTL, NK, and LAK cells and therefore might play a role in their escape from immunosurveillance. Future experiments aim to break the HLA-G-dependent tolerance of HLA-G⁺ RCC. This, together with the understanding of the underlying molecular mechanism of up-regulated HLA-G expression, may open new possibilities for the treatment of patients with RCC and have to be considered in the design of new innovative RCC-specific immunotherapies.

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REFERENCES

- Seliger, B., Maeurer, M. J., and Ferrone, S. Antigen-processing machinery breakdown and tumor growth. *Immunol. Today*, *21*: 455–464, 2000.
- Carosella, E. D., Paul, P., Moreau, P., and Rouas-Freiss, N. HLA-G and HLA-E: fundamental and pathophysiological aspects. *Immunol. Today*, *21*: 532–534, 2000.
- Ishitani, A., and Geraghty, D. E. Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens. *Proc. Natl. Acad. Sci. USA*, *89*: 3947–3951, 1992.
- Seliger, B., Abken, H., and Ferrone, S. HLA-G and MIC expression in tumors and their role in anti-tumor immunity. *Trends Immunol.*, *24*: 82–87, 2003.
- Davies, B., Hiby, S., Gardner, L., Loke, Y. W., and King, A. HLA-G expression by tumors. *Am. J. Reprod. Immunol.*, *45*: 103–107, 2001.
- Real, L. M., Cabrera, T., Collado, A., Jimenez, P., Garcia, A., Ruiz-Cabello, F., and Garrido, F. Expression of HLA G in human tumors is not a frequent event. *Int. J. Cancer*, *81*: 512–518, 1999.
- Paul, P., Cabestre, F. A., Le Gal, F. A., Khalil-Daher, I., Le Danff, C., Schmid, M., Mercier, S., Avril, M. F., Dausset, J., Guillet, J. G., and Carosella, E. D. Heterogeneity of HLA-G gene transcription and protein expression in malignant melanoma biopsies. *Cancer Res.*, *59*: 1954–1960, 1999.
- Paul, P., Rouas-Freiss, N., Khalil-Daher, I., Moreau, P., Riteau, B., Le Gal, F. A., Avril, M. F., Dausset, J., Guillet, J. G., and Carosella, E. D. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc. Natl. Acad. Sci. USA*, *95*: 4510–4515, 1998.
- Willers, J., Urosevic, M., Laine, E., Geertsen, R., Kundig, T., Burg, G., and Dummer, R. Decreased intraindividual HLA class I expression is due to reduced transcription in advanced melanoma and does not correlate with HLA-G expression. *J. Invest. Dermatol.*, *117*: 1498–1504, 2001.
- Ibrahim, E. C., Guerra, N., Lacombe, M. J., Angevin, E., Chouaib, S., Carosella, E. D., Caignard, A., and Paul, P. Tumor-specific up-regulation of the nonclassical class I HLA-G antigen expression in renal carcinoma. *Cancer Res.*, *61*: 6838–6845, 2001.
- Lefebvre, S., Antoine, M., Uzan, S., McMaster, M., Dausset, J., Carosella, E. D., and Paul, P. Specific activation of the non-classical class I histocompatibility HLA-G antigen and expression of the ILT2 inhibitory receptor in human breast cancer. *J. Pathol.*, *196*: 266–274, 2002.
- Urosevic, M., Kurrer, M. O., Kamarashev, J., Mueller, B., Weder, W., Burg, G., Stahel, R. A., Dummer, R., and Trojan, A. 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.*, *159*: 817–824, 2001.
- Wiendl, H., Mitsdoerffer, M., Hofmeister, V., Wischhusen, J., Bornemann, A., Meyermann, R., Weiss, E. H., Melms, A., and Weller, M. A functional role of HLA-G expression in human gliomas: an alternative strategy of immune escape. *J. Immunol.*, *168*: 4772–4780, 2002.
- Le Gal, F. A., Riteau, B., Sedlik, C., Khalil-Daher, I., Menier, C., Dausset, J., Guillet, J. G., Carosella, E. D., and Rouas-Freiss, N. HLA-G-mediated inhibition of antigen-specific cytotoxic T lymphocytes. *Int. Immunol.*, *11*: 1351–1356, 1999.
- Seliger, B., Hammers, S., Hohne, A., Zeidler, R., Knuth, A., Gerharz, C. D., and Huber, C. IFN- γ -mediated coordinated transcriptional regulation of the human *TAP-1* and *LMF-2* genes in human renal cell carcinoma. *Clin. Cancer Res.*, *3*: 573–578, 1997.
- Wolfel, T., Van Pel, A., Brichard, V., Schneider, J., Seliger, B., Meyer zum Buschenfelde, K. H., and Boon, T. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytotoxic T lymphocytes. *Eur. J. Immunol.*, *24*: 759–764, 1994.
- Brodsky, F. M., Parham, P., Barnstable, C. J., Crumpton, M. J., and Bodmer, W. F. Monoclonal antibodies for analysis of the HLA system. *Immunol. Rev.*, *47*: 3–61, 1979.
- Fournel, S., Huc, X., Aguerre-Girr, M., Solier, C., Legros, M., Praud-Brethou, C., Moussa, M., Chaouat, G., Berrebi, A., Bensussan, A., Lenfant, F., and Le Bouillier, P. Comparative reactivity of different HLA-G monoclonal antibodies to soluble HLA-G molecules. *Tissue Antigens*, *55*: 510–518, 2000.
- Paul, P., Rouas-Freiss, N., Moreau, P., Cabestre, F. A., Menier, C., Khalil-Daher, I., Pangault, C., Onno, M., Fauchet, R., Martinez-Laso, J., Morales, P., Villena, A., A., Giacomini, P., Natali, P. G., Frumento, G., Ferrara, G. B., McMaster, M., Fisher, S., Schust, D., Ferrone, S., Dausset, J., Geraghty, D., and Carosella, E. D. HLA-G, -E, -F Preworkshop: tools and protocols for analysis of non-classical class I genes transcription and protein expression. *Hum. Immunol.*, *61*: 1177–1195, 2000.
- Kubens, B. S., Passler, M., and Grosse-Wilde, H. Segregation study of the soluble 39-kD HLA class I heavy chain. *Hum. Immunol.*, *40*: 247–252, 1994.
- Luboldt, H. J., Kubens, B. S., Rubben, H., and Grosse-Wilde, H. Selective loss of human leukocyte antigen class I allele expression in advanced renal cell carcinoma. *Cancer Res.*, *56*: 826–830, 1996.
- Drexler, I., Antunes, E., Schmitz, M., Wolfel, T., Huber, C., Erfle, V., Rieber, P., Theobald, M., and Sutter, G. Modified vaccinia virus Ankara for delivery of human tyrosinase as melanoma-associated antigen: induction of tyrosinase- and melanoma-specific human leukocyte antigen A*0201-restricted cytotoxic T cells *in vitro* and *in vivo*. *Cancer Res.*, *59*: 4955–4963, 1999.
- Wagner, S. N., Reibmann, V., Willers, C. P., Grosse-Wilde, H., and Goos, M. Expression analysis of classic and non-classic HLA molecules before interferon α -2b treatment of melanoma. *Lancet*, *356*: 220–221, 2000.
- Stanley, A. J., Gough, M. J., Banks, R. E., Selby, P. J., and Patel, P. M. Renal carcinoma cell lines inhibit natural killer activity via the CD94 receptor molecule. *Cancer Immunol. Immunother.*, *50*: 260–268, 2001.
- Spaggiari, G. M., Contini, P., Carosio, R., Arvigo, M., Ghio, M., Oddone, D., Dondero, A., Zocchi, M. R., Puppo, F., Indiveri, F., and Poggi, A. Soluble HLA class I molecules induce natural killer cell apoptosis through the engagement of CD8: evidence for a negative regulation exerted by members of the inhibitory receptor superfamily. *Blood*, *99*: 1706–1714, 2002.