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 aberration-efficientnant tumor 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 cell populations 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 maternal-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 suggests 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 tumors 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 β2-microglobulin (17); the anti-HLA-A2 mAbs mA2.1 and BB7.2 (18); the anti-HLA-G-specific mAbs MEM-G9 (IgG1) and MEM-G1 (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.
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/0.5% BSA and 2 mM EDTA by negative selection with hapten-conjugated antibodies directed against CD3, CD14, CD19, CD36, and anti-IgG using an antihapten antibody coupled to microbeads as a secondary antibody according to the manufacturers protocol (Midtendy 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× 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 × 10⁴–1 × 10⁵ target cells labeled with 50–100 μCi ⁵¹³Iodochroman 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 × 10⁵ ³⁵Cr-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)) × 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-G9, 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, 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.

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, 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, 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, uninfected 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
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-G isofrom 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 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 HLA-G 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 to their resistance to NK and/or LAK cell-mediated lysis. However, the differential recognition of the M27237 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⁻ RCC cells (2, 4).

Because KIRs are also present on T lymphocytes, HLA-G molecules may also affect T-cell function, thereby enhancing 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 cytolysis (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


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


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