Tumor-specific Up-Regulation of the Nonclassical Class I HLA-G Antigen Expression in Renal Carcinoma

El Chérif Ibrahim, Nadia Guerra, Marie-José Terrier Lacombe, Eric Angevin, Salem Chouaib, Edgardo D. Carosella, Anne Caïgnard, and Pascale Paul


ABSTRACT

HLA-G is a nonclassical class I antigen mainly expressed at the maternalfetal interface during pregnancy where it is thought to downmodulate maternal immune response against the semiallogeneic fetus. Recent studies indicate that ectopic up-regulation of HLA-G expression on melanoma cells may also favor their escape from antitumor immune response. HLA-G expression was here investigated on paraffin-embedded tissue sections in 18 renal cell carcinoma (RCC) patients. We provide evidence that HLA-G antigen is differentially expressed in carcinoma and normal renal cells and that up-regulation of this antigen in the tumor cells is more frequent than alterations of other MHC class I or class II antigens. We also demonstrated that HLA-G cell surface expression and secretion is maintained in a tumor cell line (DM) established from an HLA-G-positive RCC lesion. Furthermore, we show that type I (α and β) and, in particular, type II (γ) IFN treatment enhance steady-state mRNA levels and cell surface expression of HLA-G in the DM cell line. As several studies suggest that HLA-G displays various functional features that allow down-modulation of immune response in vitro, we propose that selective in vitro expression of HLA-G may participate in the impairment of antitumor immunity in RCC.

INTRODUCTION

RCC, the most common type of malignant tumor in the kidney, is a refractory tumor that does not respond to conventional therapies. Nevertheless, combined IL-2 and IFN-α2a treatment induces clinical responses suggesting that the immune system is triggered and may control the growth of these tumors (1).

Like melanomas, RCC is considered as an immunogenic tumor, but RCC-associated antigens are rarely expressed limiting the vaccination trials in the patients. Although RCC contains high numbers of TILs, in vitro alterations of effector functions of these TILs have been described (2–4). Production of various immunosuppressive molecules, like IL-10, transforming growth factor-β, and gangliosides by the tumor cells (5–9), were found to play a role in the different mechanisms leading to impairment of proliferative and cytolytic activity of TILs in vivo.

It is established that the level of HLA class I and class II expression is crucial to control recognition and activation of effector functions T cells toward tumor cells. In this context, the frequent alterations of HLA class I and class II expression in tumors of various origin is one of the mechanisms that allows tumor cells to escape immune surveillance (10).

Up-regulation of expression of the nonclassical MHC class I antigen HLA-G was recently reported to modulate immune responses in pathological situations, such as cytomegalovirus infection (11), transplantation, and tumors (reviewed in Ref. 12). HLA-G is characterized by low polymorphism and by the existence of at least seven alternatively spliced mRNA transcripts that encode both membrane-bound and soluble HLA-G protein isoforms (13–15). In contrast to other MHC classical class I antigens (class Ia), HLA-G protein expression is restricted to maternofetal interface and thymus (reviewed in Ref. 16). Therefore, the involvement of HLA-G in down-modulation of the maternal immune response against the fetus was proposed (17). Recent reports demonstrated the direct interaction of HLA-G killer cell antigen with at least three (KIRD) immunoglobulin-like inhibitory receptors: ILT2/LIR-1/CD85 (18–20), ILT4 (20, 21), and KIR2DL4 (22–24). Binding of HLA-G is assumed to inhibit the cytotoxic activity of NK cells and T lymphocytes bearing these receptors. In addition, the HLA-G5 soluble protein has been shown to trigger apoptosis in activated CD8+ T lymphocytes (25). HLA-G also favors cell surface expression of another nonclassical class I antigen, HLA-E. Interaction of HLA-E with CD94/NKG2 heterodimers expressed onNK cells and subsets of CTLs results in delivery of inhibitory (NKG2-A) or triggering signals (NKG2-C), depending on the NKG2 subtype (reviewed in Ref. 26). Other roles for HLA-G in modulating cytokine production (27), angiogenesis (28), and NK cell migration (29) have been reported, indicating that up-regulation of this antigen in tumors may have various implications in the control of a tumor by the immune system.

The aim of this study was thus to investigate HLA-G expression in RCCs and adjacent normal renal tissues. Because contradictory data were reported concerning alterations of MHC antigen expression in RCC (30–38), this analysis was extended to HLA class Ia and class II antigens expression. Moreover, we here report the first evidence of an RCC-derived tumor cell line, which maintains constitutive HLA-G cell surface protein expression and secretion in culture and additionally demonstrates that IFN treatment up-regulates its HLA-G expression.

MATERIALS AND METHODS

Tissue Specimens. Tumor and adjacent normal renal samples were obtained from 18 patients with RCC who had undergone radical nephrectomy. None of the patients had received preoperative therapy. Tumors were staged according to TNM classification and examined for nuclear grade (I–IV according to Fuhrman classification). All specimens were fixed in acetic acid, formalin, and alcohol and embedded in paraffin. The tissues were cut at 4-μm thickness, mounted on pre-cleaned glass microscope slides (Menzel-Gläser, Braunschweig, Germany), deparaffinized using toluene, rehydrated through a graded series of ethanol, and rinsed in distilled water. Renal cell tumors were classified as conventional (clear cell) RCC, or papillary RCC, on the basis of findings in hematoxylin-, eosin-, and safranin-stained sections.

Cell Lines and Amniotic Fluids. Renal tumor cell lines, as well as normal renal cell primary culture, were derived from RCC and cultured as...
described previously (4). The DM RCC cell line was derived from kidney resection of patient 13 (Table 1), whereas the VM RCC cell line was obtained from an RCC patient whose lesion could not be analyzed by immunohistochemistry in this series. An EBV-transformed B-cell line established from peripheral blood lymphocytes of patient DM, LCL (DM), was cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS. The Tera-2 teratocarcinoma cells transfected either with pcDNA3.1/Hygro (+/-) expression vector alone (Invitrogen, Groningen, Netherlands) or with expression vector constructs containing cDNA of HLA-G5 and -G6 (G5pcDNA and G6pcDNA) were generated and cultured as described previously (15). The human HLA-G-positive JEG-3 chorioncarcinoma cell line (American Type Culture Collection, Rockville, MD) was cultured as specified. Amniotic fluids, used as positive controls for soluble HLA-G protein characterization, were obtained after voluntary interruption of normal pregnancies. Human recombinant IFN-α, β, and γ (IFN-α, -β, and -γ) were purchased from PeproTech (London, England) and added to cell culture medium for 48 h at defined concentrations.

**Immunocytochemistry.** Cells were cyt centrifuged on glass slides and fixed for 10 min in acetone at 4°C. Before immunohistochemistry analysis, deparaffinized tissue sections were submitted to epitope retrieval by high temperature in 10 mM sodium citrate buffer (pH 6.0) using a commercial microwave oven to optimize immunoreactivity. Slides were then rehydrated for 5 min in PBS containing 0.05% saponin and 10 mM HEPES buffer. Endogenous peroxidase activity was quenched by treating sections for 5 min at rt with 3% hydrogen peroxide in water. Nonspecific binding was prevented by blocking of sections for 10 min with 3% normal horse serum (NHS) or with a 1/10 dilution of culture supernatant) recognizing a conformational and a linear determinants were performed using exon 2-specific primer G.257F and exon 5 F and 4E: a mouse IgG2a recognizing an epitope shared by HLA-B and -C heavy chains, respectively, generously provided by S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY), B1.23.2: a mouse IgG2b recognizing an epitope on associated and on -E but not HLA-G

**Table 1** Characteristics of patients and biopsies immunostained for HLA class I and II antigen

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Histology</th>
<th>TNM classification</th>
<th>Nuclear grade</th>
<th>HLA-G (t and/or i)</th>
<th>Lack of HLA-B and -C</th>
<th>HLA-DP, -DQ, DR</th>
<th>HLA-DP, -DQ, DR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>-</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>52/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>-</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>65/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>-</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>72/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>I</td>
<td>+ (t)</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>55/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>-</td>
<td>-</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>56/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>+ (t)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>64/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>+ (t)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>46/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>+ (t)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>56/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>+ (t)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>67/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>+ (t)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>48/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>-</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>78/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>III</td>
<td>+ (e, i)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>63/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>III</td>
<td>+ (e, i)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>66/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>+ (t)</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>42/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>-</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>55/F</td>
<td>HK</td>
<td>T1N1M1</td>
<td>-</td>
<td>-</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>57/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>III</td>
<td>-</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>18</td>
<td>48/M</td>
<td>HK</td>
<td>T1N1M1</td>
<td>II</td>
<td>+ (t)</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

**HLA-G immunostaining was observed within tumour cells (t), normal epithelial tubular cells (e), and/or inflammatory infiltrating cells (i).**

**Immunoreactivity was reported within tubular cells.**

**HK, healthy kidney; cRCC, conventional (clear cell) RCC; pRCC, papillary RCC.**

**Scoring of immunohistochemical staining was performed as follows: -, no reactivity; +, staining confined to <5% of cells; ++, heterogeneous staining; +++, >90% of cells were positive.**

**Statistical Analysis.** The χ² test was used for statistical analysis; P < 0.05 were considered significant.

**Monoclonal Antibodies.** The primary antibodies used in this study were the anti-Leukocyte Common Antigen/CD45RB (clone PD7/26, mouse IgG1, 1/100 dilution; Dako), the anti-HLA-DP, -DQ, and -DR antigens (clone CR3/43, mouse IgG1, 1/50 dilution; Dako), the anti-HLA-DR antigen (clone BS.12.2, mouse IgG2b; Beckman Coulter-Immunotech, Marseille, France), the anti-CDS1 antigen (clone IC7/0A, mouse IgG1, 1/30 dilution; Dako), 87G: a purified mouse IgG2a recognizing both membrane-bound HLA-G1 and soluble HLA-G isoforms (2 and 5 μg/ml for flow cytometry and immunocytochemistry) kindly provided by D. Geraghty (Fred Hutchinson Cancer Research Center, Seattle, WA; Ref. 39), 4H84: a mouse IgG1 (1/600 dilution of asctic fluid) anti-native and -denatured HLA-G heavy chain (40) kindly provided by S. Fisher and M. McMaster (University of California, San Francisco, CA), MEM-G9 (Exbio, Prague, Czech Republic); a mouse IgG1 recognizing both membrane-bound HLA-G1 and soluble HLA-G isoforms (41), HC-10: a mouse IgG2a against a determinant expressed preferentially on β2-m-free HLA-B and -C heavy chains (42) kindly provided by H. L. Ploegh (Harvard Medical School, Boston, MA), TP25:99: a mouse IgG1 (1/2 dilution of culture supernatant) recognizing a conformational and a linear determinant on associated and on β2-m-free HLA-A, -B, -C, and -E but not HLA-G heavy chains, respectively, (43) generously provided by S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY), B1.23.2: a mouse IgG2b recognizing an epitope expressed on both β2-m-associated and -free HLA-B and -C heavy chains (44), and 4E: a mouse IgG2a recognizing an epitope shared by HLA-B and -C heavy chains (45).

**RT-PCR Analysis.** Total mRNAs were extracted using the RNA NOW reagent (Biogenex, Seabrook, TX), according to the manufacturer’s recommendations, and analyzed as described previously (46). Briefly, PCR amplifications were performed using exon 2-specific primer G.257F and exon 5–
exon6-specific primer G1004R or exon 3-specific primer G.526 and intron 4-specific primer G.41b, respectively, allowing the detection of all alternatively spliced HLA-G mRNAs and, specifically, HLA-G5 mRNA. Coamplification of β-actin cDNA was carried out in each experiment with β-actin ampliters sets (Clontech) for 16 cycles to normalize amounts of cDNAs in samples. Absence of contaminant DNA was controlled by concomitant amplification of the PCR mixture without template (H2O). PCR products were analyzed by electrophoresis in 1.5% agarose gel, and the specificity of PCR products was confirmed by Southern blot analysis using 32P-labeled oligonucleotide probe exon 3-specific G.0 or intron-4-specific G.14F. The same membranes were stripped in boiled 0.5% SDS solution and hybridized with a β-actin probe.

**Flow Cytometry.** Analysis was conducted using a FACs Vantage (Becton Dickinson) and an Epics XL (Beckman Coulter). Adherent cells were removed by trypsinization and washed in PBS containing 2% FCS. Appropriate concentrations of mAbs were added to 2.105 cells in 50 μL of PBS containing 2% FCS and incubated for 30 min at 4°C. Cells were stained with an isotype-matched irrelevant antibody to evaluate nonspecific binding. Cells were washed with PBS and incubated with phycoerythrin-conjugated goat antimouse immunoglobulin (Beckman Coulter-Immunotech) at 1/50 dilution for 30 min at 4°C. After washing and fixing in PBS containing 0.5% formaldehyde, a minimum of 5000 cells were analyzed by flow cytometry.

**Western Blot Analysis.** Cells were washed with PBS and lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40] containing protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany). After centrifugation at 15,000 × g for 30 min, supernatants (20 μL) were supplemented with 6 μL Laemmli buffer. All samples were heated for 5 min at 95°C before loading on a 12% SDS-PAGE. Proteins were then electroblotted onto polyvinylidene difluoride membrane (Hybond-P; Amersham). The filters were blocked with 5% nonfat dried milk in PBST for 1 h at room temperature and incubated with 4H84, in PBST containing 2.5% nonfat dried milk overnight at 4°C. After washing with PBST, the membranes were incubated with horseradish peroxidase-conjugated goat antimouse immunoglobulin antibody (Amersham) for 45 min at rt. After additional washes in PBST, bands were detected using Enhanced Chemiluminescence Plus reagent (Amersham).

**RESULTS**

**Immunohistochemical Analysis of HLA-G Protein Expression in RCC and Normal Kidney.** HLA-G expression was analyzed by immunohistochemistry in paraffin-embedded sections obtained from 18 randomly selected RCC patients using the HLA-G-specific 4H84 mAb (Table 1). A high incidence of HLA-G expression was observed in RCC as 11 of 18 tumors exhibited mainly a membranous/Golgi immunoreactivity with the 4H84 mAb. HLA-G expression was mostly evidenced in tumor cells (10 of the 11 positive cases, Fig. 1A) and rarely detected in inflammatory infiltrating cells (Fig. 1B); normal renal tissue removed on the same kidney at a distance from the tumor rarely expressed HLA-G (1 case, within scattered inflammatory infiltrating cells). No correlation between HLA-G expression and clinical parameters (patient’s age and sex, TNM stage) could be established from this study.

**Comparative Analysis of HLA-G versus HLA Class Ia and Class II Expression in RCC and Normal Kidney.** Concomitantly to HLA-G expression analysis, serial tissue sections were stained for HLA class Ia and class II expression (Table 1). Except for three cases, all normal components of the human nephron, such as the glomerular elements (parietal cells of Bowman’s capsule, podocytes, and mesangial cells) and all of the epithelial cells lining the proximal tubules and the distal collecting ducts, reacted positively with the anti-HLA-B and -C and HC-10 mAb. Heterogeneous MHC class I reactivity was observed, staining being more intense on the glomeruli and interstitial cells than in tubular structures. Moreover, a stronger class I HC-10 mAb staining was observed in malignant tissue in comparison with normal kidney tissue sections. The carcinoma cells exhibited a dense cell membrane staining pattern. Although use of the monomorphic HC-10 class I mAb does not allow characterization of allele- or locus-specific class I defects, total loss of class Ia antigen expression was never observed in RCC specimens. HLA class I loss foci were evidenced within a subset of epithelial cells in one RCC but were also observed in normal tissue of 3 patients (Table 1).

The HLA class II immunoreactivity evidenced with the CR3/43 mAb was more heterogeneous and was observed in both healthy and tumor tissues. Inflammatory components as well as mesangial and endothelial cells of glomeruli and intertubular capillaries were positive for expression of MHC class II antigens, whereas podocytes were negative for immunoreactivity with the CR3/43 mAb. Moreover, HLA class II antigens were detected at a significantly lower frequency in normal tubular cells (50%) than in carcinoma cells (83.3%, P < 0.05). No significant correlation could be established between the presence or lack of HLA-G expression in tumor cells and HLA class II detection in normal and tumor tubular cells.

**Characterization of Transcripts and Proteins Corresponding to Membrane-bound and Soluble HLA-G Isomers in a Renal Carcinoma-derived Cell Line.** Permanent tumor cell lines were successfully established from some RCC, and normal renal cells were obtained from normal renal tissue (4). Levels of HLA-G mRNA transcripts were analyzed in these cell lines by RT-PCR using panel HLA-G primers (G.257F/G.1004R) that detect most of the HLA-G alternative transcripts. Size discrimination of RT-PCR products corresponding to alternatively spliced transcripts that exclude one or two exons of the gene allows identification of HLA-G1, -G5, -G2 and -G4 (migrating at the same size), and -G3 transcripts. A high level of HLA-G transcripts (Fig. 2A, top panel), comparable with that observed in the JEG-3 choriocarcinoma-positive control cell line, was observed in DM tumor cell line derived from RCC 13 (Table 1), which exhibited intense HLA-G protein staining of tumor cells (Fig. 1A, e). In contrast to the high level of HLA-G transcripts detected in dissociated tumor cells (T0) and in the established DM renal carcinoma cell line, the autologous B-EBV cell line derived from patient 13 [LCL (DM)] exhibited a basal level of HLA-G transcripts. HLA-G alternative transcripts were also detected both in a normal kidney primary cell culture (PAS) and in another renal carcinoma-derived cell line (VM), but HLA-G mRNA levels were lower than those revealed in DM tumor cells, using the semiquantitative normalization with the β-actin transcripts.

Specific RT-PCR analysis of transcripts corresponding to soluble HLA-G5 reveals that soluble isoform-encoding HLA-G5 transcripts were present in DM cells both in tumor-dissociated cells and in the established tumor cell line, whereas only a very faint signal was detectable in the autologous B-EBV cells (Fig. 2A, bottom panel). Altogether, these results suggest that up-regulation of HLA-G protein expression is likely to result from specific transcriptional activation of HLA-G gene expression in the tumor cells derived from patient 13.

We then investigated if up-regulation of HLA-G transcript levels in the DM renal carcinoma cell line could be associated to cell surface expression of HLA-G1 protein in tumor cells. In accordance with the high rate of HLA-G transcripts in DM tumor cells, HLA-G1 protein expression was detected on DM cells by flow cytometry analysis using the HLA-G-specific mAbs 87G (Fig. 2B) and MEM-G9 (data not shown). In contrast, cells exhibiting lower levels of HLA-G transcripts, LCL (DM), normal kidney cells (PAS), and VM renal carcinoma cells, were devoid of HLA-G cell surface expression. Six additional renal carcinoma cell lines derived from patients that either expressed HLA-G on tumor cells 	extit{ex vivo} (patients 2, 8, and 9) or not (patients 1, 17, and 18) were negative for HLA-G cell surface expression (data not shown).

Concomitant flow cytometry analysis of class Ia HLA antigens expression in the same cell lines using B1.23.2 (HLA-B and -C alleles) and TP25.99 (HLA-A, -B, -C, and -E but not -G) mAbs revealed that these cell lines expressed classical HLA class I antigens (Fig. 2B).
A high and homogeneous HLA-G protein expression in DM tumor cell line was also confirmed by immunocytochemistry (Fig. 1A, i). Western blot analysis of HLA-G proteins using the 4H84 mAb allowed detection of specific signals corresponding to HLA-G1 and -G5 proteins in whole cell extracts from DM cells and in positive control JEG-3 cells but not in the DM (LCL) cells (Fig. 2C). Interestingly, a 30-kDa migrating band that may correspond to the HLA-G2 isoform was also observed in DM carcinoma cells.

Secretion of the soluble HLA-G protein by DM tumor cells was assessed by Western blot analysis of cultured cell supernatants (Fig. 2D).

A band of ~37 kDa, corresponding to the size of the HLA-G5 protein in HLA class I-negative Tera-2 cells transfected with an HLA-G5 expression vector, was revealed in DM tumor cells, whereas it was not detectable in DM (LCL) cells or in control Tera-2 cells transfected with empty expression vector. As expected, a high level of HLA-G5 protein was also detected in amniotic fluid used as a positive control.

IFI Treatment Enhances Both HLA Class Ia and HLA-G Cell Surface Protein Expression in the DM Renal Carcinoma Cell Line. Flow cytometry analysis of cell surface HLA-G and HLA class Ia and class II expression was conducted after 48 h of treatment of

---

Fig. 1. Immunohistochemical analysis of HLA-G antigens expression in serial sections of RCC lesions. In A, tumor cells within the RCC lesion of patient 13 (Table 1) were stained positively using the anti-HLA-G antigen 4H84 mAb (b), the anti-HLA-B and -C molecule HC-10 mAb (c), and the anti-HLA class II antigen CR3/43 mAb (d). In contrast, the immune infiltrating cells revealed with an anti-CD45RB mAb (e) and the CD31-positive capillary endothelial cells (f) were negative for HLA-G expression. In the same kidney, HLA-G antigens were not detected in normal cells (g). HLA-G expression was detected in the cytospun DM tumor cells derived from patient 13 (Table 1) using the HLA-G-specific antibody 87G (i). Isotype-matched mAbs were used as controls for nonspecific binding on RCC (a) and normal kidney (h) sections, as well as on cytospun DM RCC cells (j). In B, a papillary tumor corresponding to a kidney resected from patient 9 (Table 1) contains HLA-G-positive cells (b), identified as inflammatory infiltrating cells by their staining by anti-CD45-RB (c) and anti-HLA class II mAbs (d). All cells, including tumor cells, were stained by the anticlassical class I HC-10 mAb (e). The normal tissue resected from the same kidney was devoid of HLA-G staining (g) despite a strong staining with Leukocyte Common Antigen mAb in the numerous infiltrating cells (h). The specificity of the HLA-G stainings on these tissue sections was confirmed by the use of an irrelevant isotype-matched mAb (a and f). Original magnification: A, ×80; B, a–e, ×120; f–h, ×80.
Fig. 2. HLA-G is expressed in the DM tumor cell line (derived from RCC patient 13) as a membrane-bound and soluble protein. A, RT-PCR detection of HLA-G mRNAs in dissociated RCC lesions (70) and in the corresponding derived tumor cell lines (VM and DM). Top panel, detection of HLA-G alternative transcripts resulting from amplification with pan-HLA-G primers G.257F (exon 2) and G.1004R (exon 5/exon 6): HLA-G5, 886 bp; HLA-G1, 764 bp; HLA-G2, 488 bp; HLA-G4, 491 bp; and HLA-G3, 215 bp. Bottom panel, specific amplification of HLA-G5 transcripts corresponding to the soluble HLA-G5 protein (489 bp) with primers G.526 (exon 3) and G.4b (intron 4). HLA-G5-specific transcripts were revealed by hybridization with either the exon 2-specific G.R probe (top panel) or an intron 4-specific G.4F probe (bottom panel). Normal renal cells and the B-EBV cell line autologous to DM tumor cells, LCL (DM), were included in the analysis. Absence of contaminant DNA was controlled by concomitant amplification of the PCR mixture without a template (H2O). B, flow cytometry analysis using B1.23.2 (anti-HLA-B and -C alleles), TP25.99 (anti-HLA-A, -B, -C, and -E), and 87G (anti-HLA-G1 and -G5) mAbs depicted as filled histograms (MFI) and open histograms (mouse isotypic IgG2a control). C, whole-cell protein extracts analyzed by Western blot probing with the HLA-G-specific 4H84 mAb. Bands corresponding to HLA-G1, -G5, and the putative -G2 protein isoforms are indicated by arrows. D, Western blot analysis of HLA-G5 soluble isoform detected in DM supernatants. The Tera2-pcDNA and -G6 transfectants were used as negative controls, and Tera2-G5 transfectant cell extracts were used as a positive control for detection of soluble HLA-G proteins. Amniotic fluid (Amniot. F.438) is a physiological positive control for soluble HLA-G protein detection.

DM cells with various doses of type I (IFN-α: 200, 400, 1000, and 2500 units/ml and IFN-β: 250, 500, 1000, and 2000 units/ml) or type II IFNs (IFN-γ: 40, 80, 200, and 500 units/ml). All three IFNs used induced a 2–4-fold increase of cell surface expression of both classical class I antigens (HLA-B and -C, using the B1.23.2 and 4E mAbs) and the full-length membrane-bound HLA-G1 isoform (specifically revealed by 87G and MEM-G9 mAbs), even at the lowest concentrations of IFN used. Optimal enhancement of MHC class I antigen cell surface expression was obtained using 2500 (IFN-α), 1000 (IFN-β), and 500 units/ml (IFN-γ; Fig. 3). IFN-γ was the sole IFN to induce HLA-DR antigen expression as detected by the B8.12.2 mAb (data not shown) and is also the most effective inducer of HLA-G expression in DM cells.

A comparative RT-PCR analysis of type I and II IFN treatment revealed only a slight increase of the steady-state levels of HLA-G mRNA levels (which is more pronounced for the HLA-G5 soluble isoform encoding transcript) by IFN-γ treatment (data not shown), suggesting that posttranscriptional mechanisms may be implicated in the IFN-mediated enhancement of HLA-G protein expression in DM cells. In contrast, IFN-γ treatment of HLA-G-negative renal tumor cell lines (VM and cells derived from patients 1, 2, 8, 9, and 17) did not induce HLA-G cell surface expression (data not shown).

DISCUSSION

The major characteristic of the MHC class I HLA-G gene resides in its tissue-restricted pattern of protein expression in vivo in healthy individuals. However, recent studies indicate that the expression of this placental antigen can be activated in malignancies, including melanoma and colorectal cancer (47–49). Considering the immunomodulatory function assigned to date to HLA-G molecules, its expression in malignant cells may represent one of the various mechanisms used by tumor cells to thwart the immune response (50). Our aim was thus to evaluate HLA-G protein expression in RCC in comparison with normal matched renal tissue. HLA-G expression was detected only once within subsets of normal epithelial tubular cells. In contrast, expression of HLA-G antigens was observed in tumor cells of a significant number of RCC lesions (10 of 18), suggesting that tumor-specific activation of HLA-G expression frequently occurs in RCC malignant cells. Recruitment of patients matching that of RCC in population (age, sex, and clear cell:paillary RCC ratio) did not allow any correlation of HLA-G expression with histopathological or clinical parameters of the disease. In this series of 18 RCC specimens, 16 were of the conventional type (clear cell), whereas 2 were papillary.

Most RCC sections analyzed exhibited peripheral 4H84 immunostaining of tumor cells, compatible with a cell surface expression of HLA-G molecules. Confirming immunohistochemical analysis, the
detection of membrane-bound HLA-G1 and secreted HLA-G5 protein isoforms in tumor cells derived from an HLA-G-positive tumor strongly supports the notion that HLA-G proteins are expressed in vivo by RCC tumor cells and released as soluble factors within the tumor. As observed previously in melanoma (47), HLA-G protein expression in RCC tissue sections correlated to specific activation of HLA-G alternative transcript levels in the dissociated tumor or RCC-derived cell line. The present data showing that HLA-G expression can be maintained in vitro in an RCC-derived cell line contradicts previous reports that led to the conclusion that tumors do not express HLA-G proteins (51–53). Our previous report on melanoma biopsies and cell lines and the present study on RCC still outline that the incidence of HLA-G expression is probably higher in vivo than that detected in established tumor cell lines.

Whereas a consensus was reached concerning expression of HLA class I antigens in normal kidneys (30, 34, 35, 54), reported data concerning HLA class Ia expression in RCC tissues remains contradictory. Considering the high incidence of HLA-G expression in RCC reported in this study, the use of the W6/32 mAb that detects all β2-m-associated HLA class I antigens, including HLA-E and HLA-G, to analyze HLA class I expression in RCC may partly explain the conflicting results concerning HLA class Ia antigen expression in primary RCCs as compared with normal proximal tubules (30–34). This study is the first to attempt a combined analysis of MHC gene expression, allowing discrimination between classical and nonclassical class I antigen expression, in the same RCC patients. In contrast to W6-32 or anti-β2-m mAb, HC-10 mAb recognizes a determinant shared by HLA-B and -C heavy chains (42) and allows detection of HLA-B and -C class I loss variants in paraffin-embedded tissues but does not detect HLA-G or -E class Ib antigens. Although our analysis using HC-10 may have underestimated the detection of selective HLA-A class I or allele-specific HLA class I-negative loss variants observed previously in advanced RCC (32, 55), use of this antibody combined to HLA-G detection gives new insight for better comprehension of MHC class I alterations in tumors. Our analysis indicates that tumor-specific HLA-G class I up-regulation is a frequent event in RCC. This study also reveals that total loss of HLA-B and -C antigen expression is rarely observed in RCC and that focal loss of class I expression does not appear to be a hallmark of tumors as it is also detected in normal renal tissue.

Discrepancies were also reported in the literature concerning HLA class II detection in normal renal cells and RCC. Whereas studies described HLA-II-negative normal tubular cells (33, 35), others report heterogeneous staining in normal tissue (31, 36, 54, 56). Previous studies analyzing MHC class II antigen expression in RCC led to conflicting conclusions as a total lack of HLA class II antigens in renal carcinoma cells (33, 36), staining of 50% of RCC (31) or most if not all RCC (35, 37). Our analysis of HLA class II antigen expression, based on the reactivity of the CR3/43 mAb recognizing a shared determinant of HLA-DP, -DQ, and -DR molecules, indicates enhanced expression of MHC class II antigens in renal carcinoma as compared with normal tissues (31, 37, 38). In contrast to HLA-G up-regulation, which is only detected in the tumor tissue, MHC class II antigen expression appears less specific of the tumor site as it is also observed in normal tissue of some patients.

The mechanisms underlying up-regulation of HLA-G gene expression are poorly understood and may involve transcriptional activation by the tumor microenvironment. Factors such as stress or IL-10 have been shown to promote HLA-G gene expression (46, 57) and involve regulatory pathways that are not shared by other MHC class I genes. IFNs are potent inducers of HLA-G expression (58–61), and regulatory mechanisms that control transcriptional up-regulation of HLA-G antigens have been shown to be distinct from those implicated in up-regulation of classical MHC class I and II genes (62, 63). As expected (64), IFNs up-regulate MHC class Ia antigen expression on the DM tumor cell line. We also show that IFN-γ and, to a lesser extent, type I IFN (α and β) induced HLA-G1 surface expression in this cell line. In accordance to previous experiments using RCC cell lines (31), IFN-γ, but not type I IFNs, also induces cell surface expression of HLA-DR molecules on DM tumor cells. Levels of HLA-G5 transcripts, encoding the soluble HLA-G protein isoform, were also up-regulated upon IFN-γ treatment in this RCC cell line.

Altogether, our results indicate that HLA-G protein expression and secretion is up-regulated in a tumor-specific manner in RCCs and thus appears to be more characteristic of the malignant phenotype of renal cells than alterations of other HLA class Ia and class II antigens.

Previous description of HLA-G as a down-modulator of both NK and T lymphocyte effector cell functions in vitro allows speculation on the impact of HLA-G tumor-specific expression in modulating an efficient antitumor immune response (18–24, 65). Recent data have indeed shown that the interaction of HLA-C molecules expressed by the DM tumor cells with the p58.2 inhibitory receptor mediates inhibition of cytotoxic activity of in situ-amplified p58-positive T cells (4). Additional inhibition of cytotoxic activity by tumor-specific p58-positive T cells coexpressing ILT-2 receptor through HLA-G engagement was also investigated (data not shown). Masking of HLA-G on DM target cells or ILT-2 receptors on effector T cells did not result in additional enhancement of cytotoxicity, suggesting that the p58.2 inhibitory receptors provide the dominant inhibitory signal in these T cells (4). Lack or down-regulation of MHC class I alleles ligand of KIRs in some patients or presence of effector cell subsets that preferentially recognize HLA-G may still be of importance in vivo. Furthermore, according to previous studies, alternative roles for HLA-G, such as modulation of cytokine production (27), induction of activated T lymphocyte apoptosis (25), inhibition of NK cells migration (29), or alteration of T-cell activation by HLA-G-positive professional antigen presenting cells, may also play a role in modulating the immune response against tumors in vivo and remain to be investigated.

In addition to being an additional immune escape mechanism, aberrant activation of HLA-G in melanoma has been proposed as a rationale for the unresponsiveness to IFN-α therapy in melanoma patients (48). Our finding that IFNs also enhance HLA-G expression in some RCC tumor cells may suggest adverse effects of such treat-
ment in favoring immune responses in some patients. Characterization of HLA-G expression could thus contribute to better prediction and monitoring of RCC patients that will better benefit from IFN-α therapy.

ACKNOWLEDGMENTS

We thank M. McMaster, S. Fisher, D. Geraghty, S. Ferrone, and H. L. Ploegh for the kind gift of antibodies and L. Gourand for providing amniotic fluid. We also thank J.-A. Gavign for editing the manuscript. Finally, we thank E. Savariou, R. Nancel, and B. Boursin for photographic work.

REFERENCES


HLAG EXPRESSION IN RENAL CELL CARCINOMA


Tumor-specific Up-Regulation of the Nonclassical Class I HLA-G Antigen Expression in Renal Carcinoma

El Chérif Ibrahim, Nadia Guerra, Marie-José Terrier Lacombe, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/18/6838

Cited articles
This article cites 63 articles, 25 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/18/6838.full.html#ref-list-1

Citing articles
This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/61/18/6838.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.