Altered Pattern of Major Histocompatibility Complex Expression in Renal Carcinoma

Tumor-Specific Expression of the Nonclassical Human Leukocyte Antigen-G Molecule Is Restricted to Clear Cell Carcinoma While Up-Regulation of Other Major Histocompatibility Complex Antigens Is Primarily Distributed in All Subtypes of Renal Carcinoma

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Renal epithelial cancers represent a heterogeneous group of neoplasms arising from the malignant transformation of presumed diverse cell lineages. We recently demonstrated that tumor-specific up-regulation of human leukocyte antigen (HLA)-G, a nonclassical HLA class Ib molecule that might be involved in immune evasion by tumor cells, frequently occurs in conventional (clear cell) renal carcinoma. We here examined whether HLA-G activation is a common process affecting all types of renal epithelial tumors. We analyzed a series of 38 paraffin-embedded tumors including clear cell, papillary, chromophobe, collecting duct carcinoma, and oncocytoma. Seven of 12 (58%) clear cell tumors were positive by immunohistochemistry, whereas all of the other subtypes of renal carcinoma were negative for HLA-G expression. Developing or adult normal renal tissue were devoid of HLA-G expression. We also observed that ectopic expression of HLA class II antigens occurs more frequently in clear cell renal carcinoma than in other subtypes of renal tumors. Moreover, in contrast to the common observation of a down-regulation of major histocompatibility complex class Ia antigens reported in various tumors, the concomitant study of the same biopsies for classical HLA class Ia antigen expression revealed a general increase of HLA class Ia expression, regardless of histological subtypes. These results provide evidence for the heterogeneity of major

histocompatibility complex expression patterns in renal carcinoma and support the hypothesis that specific mechanisms underlying the malignant transformation into clear cell renal carcinoma up-regulate expression of HLA-G and to a lesser extent HLA class II molecule expression. Considering the immunotolerant role of HLA-G toward the immune response, these mechanisms may thus provide renal cell carcinoma tumor cells with additional means to escape immune surveillance. (*Am J Pathol* 2003, 162:501–508)

Unlike the widely expressed classical human leukocyte antigen class Ia molecules (HLA class Ia), HLA-A, HLA-B, and HLA-C, the nonclassical HLA Ib molecule, HLA-G, exhibits a physiological expression mainly restricted to the placental tissues, thymic epithelial medulla, peripheral blood, and amniotic fluid.^{1,2} The expression of HLA-G molecules on extravillous cytotrophoblast, which lacks HLA-A and HLA-B antigen expression, has led to the concept that HLA-G might contribute to the maternal-fetal tolerance.³ This idea was further strengthened by *in vitro* evidence that HLA-G molecules interact with the inhibitory receptors p49/KIR2DL4/CD158d and ILT2/LIR1/CD85j found on natural killer (NK) and T cells, thus lowering the cytotoxic activity of these effector cells.^{4–9} In addition, HLA-G may modulate antigen presentation by other immune effector cells by interacting with inhibitory

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receptors such as ILT2/LIR1/CD85j and ILT4/CD85d, expressed on B lymphocytes, monocytes, and dendritic cells.^{10–12} Moreover, HLA-G leader peptides can favor cell surface expression of another HLA class Ib molecule, HLA-E, which is the specific ligand of both triggering and inhibitory CD94/NKG2 heterodimeric receptor expressed by all NK cells and subsets of T cells.^{13,14}

Alteration of HLA class Ia and II antigen expression frequently occurs in cancer and is considered to favor tumor escape from immune surveillance.¹⁵ Recently, we and others have detected HLA-G molecules *ex vivo* in a variety of malignancies in which down-regulation of HLA class Ia antigens is also commonly reported, including melanoma, breast, and lung cancers and cutaneous lymphoma.^{16–20} We have previously reported that HLA-G expression in renal cell carcinomas (RCCs) was exclusively restricted to tumor cells.²¹ Constitutive and interferon-inducible cell surface expression of HLA-G proteins was also demonstrated *in vitro* on primary cultured thymic or amniotic epithelial cells²² and on a renal carcinoma cell line derived from an HLA-G-positive tumor biopsy.²¹ Collectively, these results suggest that HLA-G molecule expression might be involved in the impairment of the anti-tumor immunity or resistance to immunotherapeutic approaches such as interferon- α .

Adult RCCs consist of a heterogeneous group of tumors with distinct clinical, histopathological, and genetic features.^{23–25} In our initial survey, most renal carcinomas analyzed were of the conventional (clear cell) type of renal carcinoma.²¹ Therefore, the present study aimed at determining if HLA-G, as well as other HLA class Ia and II molecules, exhibit specific patterns of expression in the most common morphotypes of renal epithelial neoplasms. We therefore performed immunochemistry analysis of major histocompatibility complex (MHC) antigen expression in a series of paraffin-embedded lesions including conventional (clear cell) (accounting for ~75 to 80% of all RCCs), papillary (~10 to 15%), and chromophobe (~5%) RCC, collecting duct carcinoma (<1%), and oncocytoma (which are considered as benign renal neoplasms).²³

Furthermore, as HLA molecule expression within the tumors could be related to the histogenesis and/or reflect activation of oncofetal or embryonic genes, we investigated physiological HLA antigen expression in developing and adult normal renal tissue. We here report that HLA-G expression is strictly restricted to the clear cell-type RCCs, whereas global HLA class Ia expression appears up-regulated in most renal tumors regardless of their histological subtype.

Materials and Methods

Tissue Specimens

Tumor and adjacent normal renal samples were obtained from 38 patients who had undergone radical nephrectomy (Tenon Hospital, Paris, France). None of these cases belonged to the previous published study.²¹ None of the patients had received preoperative therapy. Em-

bryonic and fetal kidneys were retrieved from therapeutic abortion material conserved in the archives of the Department of Pathology (Tenon Hospital, Paris, France); a microscopic examination confirmed that renal tissue was morphologically normal. All tissues were cut at 4 μ m thickness, and mounted on precleaned glass microscope slides (Menzel-Gläser, Braunschweig, Germany). Renal cell tumors were identified as conventional (clear cell) (ccRCC, $n = 12$), papillary carcinoma (pRCC, $n = 10$), chromophobe (chRCC, $n = 7$) RCC, collecting duct carcinoma (Bellini, $n = 4$), or renal oncocytoma ($n = 5$) according to the revised classification of renal tumors.^{23,24} Tumors were staged according to tumor-node-metastasis (TNM) classification and examined for nuclear grade (I to IV according to Fuhrman classification).^{26,27}

Antibodies

The primary monoclonal antibodies (mAbs) used in this study were the anti-leukocyte common antigen/CD45RB (clone PD7/26, 1:100 dilution; DAKO, Glostrup, Denmark); the anti-HLA-DP, anti-HLA-DR, and anti-HLA-DQ antigens (clone CR3/43, 1:50 dilution; DAKO, Denmark); the anti-gp100/Pmel17 (clone HMB45, 1/50 dilution; DAKO, Carpinteria, CA); 4H84, an IgG1 (1:500 dilution of ascitic fluid) anti-native and denatured HLA-G heavy chain²⁸ kindly provided by M. McMaster (University of California, San Francisco, CA); HC-10, an anti-determinant preferentially expressed on β_2 m-free most HLA-B and HLA-C and some HLA-alleles of HLA class Ia heavy chains²⁹ kindly provided by H. L. Ploegh (Harvard Medical School, Boston, MA). The NCAM-L1 (clone C-20, 1:150 dilution), a goat polyclonal antibody raised against a peptide mapping to the carboxy terminus of human anti-L1-CAM (Santa Cruz Biotechnology, Santa Cruz, CA) was used in double-staining experiments.

Immunohistochemistry

Immunostainings using mouse mAbs as a first antibody were conducted as previously described.²¹ Negative controls of immunostaining procedures were obtained by using the HMB45 mAb as a primary antibody. For double staining with anti-L1-CAM antibody and HC-10, deparaffinized slides were incubated for 30 minutes with the NCAM-L1 antibody diluted in Tris-buffered saline (pH 7.5) with 0.1% Tween-20, 3% albumin, and 10% rabbit normal serum (DAKO), followed by sequential incubations with rabbit anti-goat biotinylated antibody (1:400 dilution, DAKO) and alkaline phosphatase-conjugated streptavidin (1:250 dilution, DAKO). The immunoreaction was visualized with developing solutions containing blue-purple 5-bromo-4-chloro-3-indoxyl phosphate with nitroblue-tetrazolium-chloride (BCIP/NBT) supplemented with 1 mmol/L of levamisole (to quench endogenous alkaline phosphatase activity). To block antibody cross reactivity and retrieve epitopes, the slides were microwaved twice for 5 minutes at 360 W.³⁰ The sections were then processed on an automated instrument (Ventana Nexes; Ventana Medical Systems, Paris, France) for the HC-10

immunostaining (1:1000 dilution) using an indirect biotin avidin system, the Ventana Basic DAB detection kit (Ventana Medical Systems) according to the manufacturer's instructions. Finally, sections were dehydrated and mounted in Eukitt.

Statistical Analysis

HLA class Ia, class Ib (HLA-G), and class II expression was compared between the groups classified according to the histological subtype, the TNM stage, or the nuclear grade, using chi-square test and the Fisher's exact *t*-tests.

Results

Tumor-Specific Induction of HLA-G Molecule Expression in Renal Carcinoma Is Specific to the Clear Cell-Type Tumor Cells

Immunohistochemical analysis of a series of 38 renal carcinoma and adjacent healthy tissues revealed that HLA-G protein expression was exclusively detected in tumor cells, both with a membrane and to a less extent a cytoplasmic staining. Of note, all CD45RB-positive inflammatory cells were negative for HLA-G expression (data not shown). Moreover, HLA-G protein expression was found in the clear cell subtype of carcinoma but never detected in papillary RCC, chromophobe RCC, collecting duct carcinoma, and oncocytoma (Figure 1A and Figure 2). This selective activation of HLA-G antigen expression occurred in 7 of 12 cases (58.3%) independent of the age and gender of patients as well as the nuclear grade and the size of tumors (Table 1).

Expression of HLA Class Ia Antigens Is Increased in Most Types of Renal Tumor Cells While Expression of HLA Class II Antigens Is More Frequent in the Clear Cell Type

Concomitant with HLA-G expression analysis, serial tissue sections were stained for HLA class Ia and II expression (Table 1). More pronounced anti-HLA class Ia staining was always observed in tumor cells, as compared with normal adjacent tubule cells. This unexpected HLA class Ia expression pattern was detected in all subtypes of renal carcinoma analyzed, with the exception of chromophobe carcinoma (Table 1 and Figure 2). In addition, total HLA class Ia antigen loss, frequently reported in various types of tumors, was never observed in our series. HLA class II immunoreactivity could be observed in tumor cells in each histological group of renal carcinoma. Nevertheless, HLA class II-induced expression in tumor cells was significantly more frequent in clear cell renal carcinoma (Figures 1A and 2).

Discrimination of Normal Epithelial Renal Components by HLA Ia Antigen Expression

HLA-G expression was undetectable in the normal renal tissue adjacent to the tumor, in particular in the proximal tubules from which clear cell RCC is supposed to originate. Of interest, the detailed analysis of HC-10 mAb reactivity on normal kidney tissues revealed a heterogeneous pattern of staining. Mesangial and endothelial cell membranes were intensely stained for HLA-B and HLA-C antigen expression, whereas the immunostaining was cytoplasmic in tubules, often very low or negative in proximal convoluted tubules, slightly increased in distal tubular cells, and moderate to high in some cells of connecting tubules and the collecting duct. In these latter structures, we observed an alternation of HLA Ia-positive and -negative antigen expression, which is reminiscent of the alternation pattern of intercalated and principal cells described in these segments.³¹ The double staining of normal adult kidney tissues with HC-10 mAb and an anti-L1-CAM polyclonal antibody that is specific to an adhesion molecule basolaterally expressed only by principal cells,^{31,32} showed that expression of HLA class Ia antigens and L1-CAM molecules is mutually exclusive, L1-CAM-negative cells being stained for HLA class Ia antigens and corresponding to intercalated cells (Figure 1B). HLA class II expression was mainly observed in mesangial and endothelial cell membranes of glomeruli and juxtatubular capillaries within normal kidney tissues, and weakly in rare dispersed tubular cell cytoplasm, in proximal, distal, and connecting tubules and in collecting ducts.

HLA-G Molecules Are Not Expressed in the Developing Normal Kidney

To explore HLA antigen expression during embryonic and fetal kidney morphogenesis, biopsies from developing kidneys at different ages were analyzed by immunohistochemistry (8, 10, 17, 18, 25, and 27 weeks of gestation). HLA-G molecules were never detected in developing kidney whereas HLA-B and HLA-C antigens were expressed as early as 8 weeks within endothelial cells, and appeared in epithelial segments by 17 weeks. As observed in adult kidney, HLA class Ia antigen expression is restricted to a minority of collecting duct cells (Figure 1C). At this stage of development (17 to 18 weeks of gestation) the differentiation between principal *versus* intercalated cells is not achieved. Nevertheless HC-10-positive cells possess a smaller cytoplasm than the HC-10-negative cells within which they seem to intercalate (Figure 1C, right enlargement). HLA class II antigens were almost exclusively expressed by endothelial cells but not by epithelial cells in the developing kidney.

Discussion

Adult RCC is a challenging model for understanding the mechanisms involved in tumor escape from immune sur-

A

4H84 (HLA-G)

HC-10 (HLA-B,C)

**CR3/43
(HLA-DP,DQ,DR)**

Clear Cell RCC

Papillary RCC

Chromophobe RCC

Bellini Duct Carcinoma

Oncocytoma

B

C

Figure 2. Rate of HLA-G, HLA-B, and HLA-C and HLA II antigen immunodetection in tumor cells in a series of paraffin-embedded renal carcinomas corresponding to clear cell (cc), papillary (p), chromophobe (ch), collecting duct (B), and oncocytoma (o) subtypes. Statistical significance of the differential expression in one subtype of carcinoma as compared to the other type of tumors is indicated above the histograms.

veillance. In a previous study, we reported that clear cell RCC displayed an ectopic HLA-G expression pattern, which suggested that HLA-G could participate in the impairment of anti-tumoral immunity observed during the course of RCC. Here we investigated HLA-G expression in an enlarged series of RCCs, including different histological subtypes (clear cell, papillary, chromophobe, and collecting duct types) in comparison with HLA class Ia and HLA II expression, to determine whether a common or specific pattern of HLA expression could be associated with the different subtypes of RCC. HLA-G antigens were expressed in nearly 60% of clear cell renal carcinomas but not on matched healthy kidneys, confirming our previous data.²¹ Surprisingly, these molecules were absent from the other subtypes of renal carcinoma. We thus hypothesize that one of the specific mechanisms underlying the malignant transformation into clear cell carcinoma controls one or several genes directly involved in the regulation of the HLA-G protein expression. In addition, ectopic HLA-G expression in clear cell carcinoma does not appear to be the result of a process reactivating expression of genes that are expressed in kidney during fetal development because no HLA-G molecules were detected in any embryonic or fetal kidney at various steps of organogenesis. Furthermore, up-regulation of HLA-G expression could not be correlated with the stage or the grade of the tumor favoring the view that induction of HLA-G molecule expression is concomitant to malignant dedifferentiation. Of note, Urosevic and colleagues^{18,19} recently reported that HLA-G up-regulation in lung carcinoma and cutaneous lymphoma was related to interleukin-10 expression, and proposed that an auto-crine pathway of immune response abrogation, through induction of interleukin-10 and HLA-G. Nevertheless, no significant interleukin-10 expression could be detected

by immunohistochemistry in the HLA-G-positive or -negative clear cell RCC cases of the present series (data not shown).

In lung cancer and melanoma, HLA-G expression in tumor cells was reported to be associated with focal or complete HLA class Ia antigen loss, suggesting that in these cases HLA-G could protect HLA class Ia-deficient tumoral cells from NK-mediated cell lysis^{17,18} or participate in the inhibition of cytotoxic responses of T cells expressing HLA-G receptors. In the present RCC series, this HLA class Ia-/HLA-G⁺ pattern was not observed. In contrast to previous studies reporting HLA class Ia selective loss in advanced RCC,^{33,34} our observation is that classical HLA class I are expressed at a higher level in most tumor cells, as compared to their normal counterparts. These results, suggest that classical HLA class I loss of expression is unlikely to explain the aggressiveness of RCC, and are in agreement with the previous findings of an increase of HLA class Ia antigens expression in renal carcinoma using immunohistochemistry,^{21,34-39} and the alterations of effector functions of the renal carcinoma-infiltrating lymphocytes.⁴⁰⁻⁴³

The independence of HLA class Ia and HLA-G expression in this RCCs series was underscored by the fact that HLA class Ia was up-regulated, regardless of the histological subtype. Thus, the anti-HLA Ia immunostaining patterns could not argue for or against the theory that chromophobe RCC represents a malignant transformation in oncocytomas.

Of interest, we noted that in normal renal tubules HLA-Ia antigens were mainly expressed by a subpopulation of cells located in connecting tubules and in the collecting duct. The exclusive staining of HLA class Ia and L1-CAM (a specific marker of the principal cells in kidney^{31,32}), strongly suggests that HLA class Ia antigens are predominantly expressed in intercalated cells within normal renal tissue, with a possible ancillary function in differentiation and cell adhesion as previously proposed.^{44,45} Even though the significance of this HLA class Ia expression in intercalated cells may need to be further determined, this specific expression in intercalated cells was not conserved during tumorigenesis, as we observed HLA class I antigen expression in histological subtypes that originate from different segments of the nephron. Notably, the mutually exclusive expression of L1-CAM and HLA-B and HLA-C in normal kidneys was not conserved in carcinoma because some RCCs of our series could co-express these molecules (Table 1).

Furthermore, confirming microarray data,⁴⁶ we found that clear cell carcinoma is distinct from the other subtypes of renal carcinoma by its ability to more frequently express HLA class II antigens. Because some genetic

Figure 1. Immunohistochemistry analysis of fetal and adult normal kidney and renal carcinoma. **A:** Serial labeling of five subtypes of renal epithelial neoplasms. HLA-G staining is positive in clear cell carcinoma and negative in other subtypes. HLA class Ia staining is positive in all subtypes, with a predominant membrane pattern of staining. HLA-DP, HLA-DQ, and HLA-DR staining of tumor cells is more frequently encountered in clear cell carcinoma. HLA class II antigen expression is detected in inflammatory cells but not in tumor cells in shown cases of papillary and chromophobe RCC. **B:** Double staining of adult normal kidney section with anti-L1-CAM (blue chromogen) and anti-HLA-B and anti-HLA-C antigens (brown chromogen). **Arrows** indicate HLA-Ia-positive intercalated cells. Of note, endothelial and mesangial cells of the glomerulus (gl) are exclusively immunostained for HLA-B and HLA-C antigens whereas principal cells of connecting tubules/collecting duct (cd) are exclusively L1-CAM-positive. In contrast, proximal convoluted tubular cells (pt) are negative for both HLA class Ia and L1-CAM antigen expression. **C:** Cortical localization of a 17-week-old fetal kidney immunostained with anti-HLA-B and anti-HLA-C mAbs (red chromogen). **Right:** Enlargement of central image focusing on the heterogeneous and lateral staining of collecting duct cells.

Table 1. Comparative Immunohistochemical Analysis of HLA-G, HLA-Class Ia and HLA Class II Molecule Expression in a Series of Renal Carcinomas

Patient no.	Age/sex	Histology	TNM classification	Nuclear grade*	HLA-G	HLA-B and -C†	HLA-DP, -DQ, -DR†
1	65/M	HK			—‡	+/-	—
		ccRCC	T2	II	++	++	—
2	76/F	HK			—	+/-	—
		ccRCC	T1	II	—	+++	—
3	54/M	HK			—	+/-	—
		ccRCC	T2	II	+	+++	+/-
4	69/M	HK			—	+/-	+/-
		ccRCC	T1	III	+++	++	—
5	56/M	HK			—	+/-	—
		ccRCC	T1	III	+++	+++	+/-
6	77/M	HK			—	+/-	+/-
		ccRCC	T1	III	+	+++	+
7	54/F	HK			—	+/-	—
		ccRCC	T2	III	+	+++	+/-
8	58/M	HK			—	+/-	—
		ccRCC	T3b	III	—	+	+/-
9	48/M	HK			—	++	—
		ccRCC	T3b	III	—	+++	++
10	68/M	HK			—	++	+/-
		ccRCC	T3b	IV	—	+++	+
11	51/M	HK			—	+	+/-
		ccRCC	T3	IV	+++	+++	+++
12	57/M	HK			—	—	—
		ccRCC	T3b	IV	—	++	—
13	33/M	HK			—	+/-	—
		pRCC	T1	II	—	+++	—
14	56/M	HK			—	+	+/-
		pRCC	T1	III	—	+++	—
15	55/M	HK			—	+/-	—
		pRCC	T2	III	—	+++	—
16	73/M	HK			—	+/-	+/-
		pRCC	T1	III	—	+	—
17	53/M	HK			—	++	+
		pRCC	T1	III	—	+++	+
18	56/M	HK			—	+++	—
		pRCC	T1	III	—	+++	—
19	38/M	HK			—	+	+/-
		pRCC	T2	IV	—	+++	+
20	48/M	HK			—	+/-	+/-
		pRCC	T2	III	—	+++	—
21	65/M	HK			—	+/-	—
		pRCC	T1	II	—	+++	—
22 [§]	51/F	pRCC	T1	IV	—	+	—
23	63/M	HK			—	+/-	—
		chRCC	T1	II	—	+/-	—
24	34/M	HK			—	+/-	—
		chRCC	T2	II	—	+/-	—
25	53/F	HK			—	+/-	+/-
		chRCC	T3a	IV	—	+++	+
26 [§]	62/F	chRCC	T3a	IV	—	+/-	++
27 [§]	NA	chRCC	T3	III	—	+/-	+/-
28 [§]	NA	chRCC	T3	III	—	+++	—
29 [§]	NA	chRCC	T3	III	—	+	—
30	NA	HK			—	—	—
		Bellini	T3	IV	—	+	+/-
31	NA	HK			—	+/-	—
		Bellini	T3	IV	—	+	—
32 [§]	NA	Bellini	T3	IV	—	+++	+++
33 [§]	NA	Bellini	T3	IV	—	+++	—
34	84/M	HK			—	+/-	—
		Oncocytoma			—	+	—
35	62/M	HK			—	+/-	+/-
		Oncocytoma			—	+++	—
36	65/M	HK			—	+/-	+
		Oncocytoma			—	+++	—
37	67/F	HK			—	—	—
		Oncocytoma			—	+++	—
38	53/F	HK			—	+	—
		Oncocytoma			—	++	+/-

*According to Fuhrman classification.

†Immunoreactivity confined to epithelial tubular or tumoral cells is reported.

‡Scoring of immunohistochemical staining was performed as follows: —, no reactivity; +/-, staining confined to <5% of cells; +, staining comprised 5–20% of cells; ++, staining comprised 20–90% of cells; +++, >90% of cells were immunoreactive.

§Healthy kidney was not available for analysis. NA: not available, HK: healthy kidney, ccRCC: clear cell renal carcinoma, pRCC: papillary renal carcinoma, chRCC: chromophobe renal carcinoma, ND: not determined.

regions are preferentially mutated or rearranged during clear cell carcinoma genesis,⁴⁷ it will be of further interest to test whether one of the genes contained in these genetic regions could coordinate HLA-G and/or HLA II gene up-regulation while not affecting expression of HLA class Ia antigens.

In conclusion we here provide evidence for RCC diversity in terms of immune-related molecule expression such as HLA-G and to a lesser extent HLA class II. Further investigations will be necessary to determine the precise impact of HLA-G expression with regards to tumor progression and clinical outcome of the disease in patients. Better knowledge of the specific features controlling the relative HLA expression patterns in tumors may nevertheless provide insights for a better targeting of immunotherapy protocols in accordance to the subsets of HLA molecules expressed *in vivo* in the tumor subtypes.

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