Heterogeneity of HLA-G Gene Transcription and Protein Expression in Malignant Melanoma Biopsies

Pascale Paul, Francisco Adrián Cabestró, Frédérique-Anne Le Gal, Iman Khalil-Daher, Caroline Le Danff, Michel Schmid, Sylvaine Mercier, Marie-Françoise Avril, Jean Dausset, Jean-Gérard Guillet, and Edgardo D. Carosella


ABSTRACT

Nonclassical MHC class I HLA-G antigen expression is tissue specific and is thought to play a role in tolerance of the semiallogeneic fetus by the maternal immune system. Ectopic expression of HLA-G by tumor cells provides them with an additional mechanism of escape from immunosurveillance by host cytotoxic effector mechanisms. The aim of this study was to assess the expression of nonclassical HLA-G antigens in ex vivo human melanoma biopsies.

HLA-G mRNA levels corresponding to both membrane-bound and soluble protein isoforms were analyzed in tumor specimens obtained from primary or metastatic melanomas of 23 patients. High levels of HLA-G transcription were detected in tumor specimens in 5 of 23 patients and found to be comparable in both lymph node and skin metastases. HLA-G mRNA transcript levels at tumor sites in 18 of these patients were compared with those in samples of their own healthy skin and were higher in the tumor tissue in 12 patients. Differential expression of mRNA transcripts corresponding to soluble and membrane-bound HLA-G was also observed in some tumor biopsies. HLA-G protein expression was detected in tumors that exhibited high levels of HLA-G transcription by immunofluorescence of frozen sections and Western blot analysis of both tumor and healthy skin biopsies, using anti-HLA-G-specific monoclonal antibodies. This work provides evidence that HLA-G gene transcription and protein expression can be up-regulated ex vivo in melanoma. Our finding that several of the tumors studied expressed high levels of HLA-G provides additional clues as to how a tumor can be selected in vivo to escape from cytotoxic antitumor responses, constituting a new parameter to be considered in the design of therapeutic approaches aimed at enhancing antitumor immune responses.

INTRODUCTION

Several studies have highlighted the importance of an effective T-cell immune response against specific human melanoma-associated antigens. Most tumors are still able to escape cell-mediated immune responses, progressing into metastatic disease. Several explanations have been proposed as to why the T cell-mediated immune response is ineffective in eliminating melanoma cells, including defective helper T-cell function, inadequate costimulation of T cells, and deficient or reduced expression of MHC class I molecules caused by a defect in the class I heavy chain, the β2-microglobulin light chain, or the TAP peptide transporter (1–4). Melanoma therapies aimed at enhancing tumor immunogenicity have recently been the subject of an increasing amount of interest (5, 6). This work provides evidence that HLA-G gene transcription and protein expression can be up-regulated ex vivo in melanoma. Our finding that several of the tumors studied expressed high levels of HLA-G provides additional clues as to how a tumor can be selected in vivo to escape from cytotoxic antitumor responses, constituting a new parameter to be considered in the design of therapeutic approaches aimed at enhancing antitumor immune responses.

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2 To whom requests for reprints should be addressed, at DSV-DRM, SRH/CEA, Commissariat à l’Energie Atomique, Centre Hayem, Hôpital Saint-Louis, 1 avenue Claude-Vellefaux, 75010 Paris, France. Phone: 33 (0)1 53 72 21 42; Fax: 33 (0)1 48 03 19 60; E-mail: paul@dsuff.cea.fr.

3 The abbreviations used are: NK, natural killer; KIR, killing inhibitory receptor; RT-PCR, reverse transcriptase-PCR; mAb, monoclonal antibody.
vessel endothelial cells (23), and IFN-γ-treated mononuclear phagocytes (24). The HLA-G molecule exhibits very limited polymorphism and is thought to be able to interact with several described KIRs of the immunoglobulin superfamily including p49 (25), ILT2 and ILT4 (26, 27), expressed at the surface of NK cells but also on T-cell subsets, dendritic cells, B cells, and monocyte lineages. Direct interaction of HLA-G with the widely expressed CD94/NKG2A KIR receptors of the C-type lectin superfamily has been recently refuted (28, 29), with the demonstration that the nonclassical HLA-E molecule is the ligand for this receptor. The HLA-G peptide leader, like other class I peptides, allows HLA-E expression at the cell surface and can, thus, indirectly favor such interactions. The capacity of HLA-G to function as an antigen that can inhibit cytotoxic NK effector function of the maternal immune system against its semiallogeneic fetus (30) has led as an antigen that can inhibit cytotoxic NK effector function of the maternal immune system against its semiallogeneic fetus (30) has led us to investigate how ectopic expression of HLA-G in tumors could favor escape of HLA-G-expressing tumor cells from immunosurveillance (19). To further evaluate the biological relevance for patients of our previous observation of HLA-G expression in melanoma cell lines and to confirm that HLA-G is, indeed, detectable in vivo, we studied the biological relevance for patients of our previous observation of HLA-G expression in melanoma cell lines and to confirm that HLA-G is, indeed, detectable 

### Table 1: Clinical and histopathological characteristics and levels of HLA-G transcripts encoding membrane-bound and soluble isoforms of surgically removed biopsies of 23 melanoma patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>B index</th>
<th>Status</th>
<th>Tissue</th>
<th>G1/G5</th>
<th>G2/G4</th>
<th>G3</th>
<th>G.526–G.i4b</th>
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<tr>
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<td>+/−</td>
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<tr>
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<td>DOD</td>
<td>SM</td>
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<td>+/−</td>
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<td>−</td>
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<td>SPT</td>
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<td>++++</td>
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</tbody>
</table>

**Notes:**
- **B index:** Breslow’s index; **HS:** healthy skin; **SPT:** skin primary tumor; **HT:** healthy s.c. tissue; **SM:** skin metastasis; **HLN:** healthy lymph node; **LNM:** lymph node metastasis; **R:** regression; **NED:** no evidence of disease; **AWD:** alive with disease; **DOD:** died of disease.
- **PCR (G.257–G3.U):** indicates intensity of bands corresponding to the size of HLA-G1/G5, G2/G4, G3 transcripts as detected by RT-PCR, using primers that detect all HLA-G alternative transcripts. **G.526–G.i4b:** corresponds to specific amplification of the HLA-G5-soluble isoform.

**Abbreviations:**
- **HU:** hematological unit; **HS:** healthy skin; **SPT:** skin primary tumor; **HT:** healthy s.c. tissue; **SM:** skin metastasis; **HLN:** healthy lymph node; **LNM:** lymph node metastasis; **R:** regression; **NED:** no evidence of disease; **AWD:** alive with disease; **DOD:** died of disease.

**Material and Methods**

**Patients and Tissue Samples.** From 1997 through 1998, patients undergoing surgery for melanoma in the Gustave Roussy Institute, Villejuif, France, were asked to participate in a prospective study approved by the local ethics committee (Comité Consultatif de Protection des Personnes pour la Recherche Biomédicale). Patients with primary melanoma were included only if the size
of their tumor was large enough to adequately assess Breslow’s thickness and to provide a sample for the study. Small-sized primary tumors were excluded. For skin and regional lymph node metastases, samples were provided by the pathologist if tissue samples were large enough to assess diagnosis and resection quality. After giving informed consent, 23 consecutive patients undergoing surgery at the Institut Gustave Roussy were included in the study. No patient had received chemotherapy or systemic treatment during the 6 months prior to participation in the study. At the time they were included in the study, eight patients had an isolated primary melanoma, two patients had local recurrences within a 5-cm radius of the resected primary lesion, three patients had multiple skin metastases on the legs, eight patients had regional lymph node metastasis (including one who had an unresected primary tumor and another with in-transit metastasis), and two patients had disseminated disease for which they had undergone diagnostic (an untreated patient) or palliative treatment. From these patients, samples were removed from tumors under aseptic conditions, frozen in liquid nitrogen, and stored until RT-PCR and immunohistochemical staining could be carried out. Frozen blocks were cut into 5-μm sections, mounted on slides, and stained by the H&E method to evaluate the presence of tumor. Immunoperoxidase staining with S-100 and HMB45 antibodies (DAKO, Glostrup, Denmark) was conducted using an ultraturrax device (IKA Labortechnic, Staufen, Germany) and by centrifugation at 10,000 × g for 20 min, the protein concentration was determined using a Bio-Rad (Richmond, CA) DC protein assay kit. Twenty-five-μg aliquots of total protein were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Hybond N+, Amersham). Hybridization was carried out with the HLA-G-specific G.R (5′-GGT CAG TTG CTT CAT TCT GTC) and G.14F (5′-GAG GCA TCA TGT CTG TTA GG) probes, as described previously (19).

**Immunofluorescence Analysis of Tumor Biopsies.** Standard methods were used to carry out immunohistochemistry on frozen sections that had been fixed in acetone for 8 min and then rinsed in PBS and blocked in normal rabbit serum (DAKO) in PBS for 10 min. Samples were incubated with primary antibody for 1 h at room temperature, followed by incubation with a secondary FITC-conjugated antiserum immunoglobulin antibody for 15 min. Sections were counterstained with 4′,6-diamidino-2-phenylindole detection. A Nikon Diaphot 300 inverted microscope and a Nikon planochromat ×60 objective lens with a numerical aperture of 1.4 (Nikon Corp., Melville, NY) were used for fluorescence microscopy. The following antibodies were used: anti-MHC class I W6/32 (DAKO), antihuman melanoma HMB45 (DAKO), and anti-HLA-G 87G (which detects the 5′-GAG GCA TCA TGT CTG TTA GG) probes, as described previously (19).

**Western Blot Analysis of HLA-G Proteins in Tumor Samples.** When enough tumor specimen was available, proteins were extracted from melanoma tissues by homogenization in lysis buffer [25 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1% NP40, and protease inhibitors (Complete; Boehringer Mannheim)], using an ultraturrax device. After removal of insoluble material by centrifugation at 10,000 × g for 20 min, the protein concentration was determined using a Bio-Rad (Richmond, CA) DC protein assay kit. Twenty-five-μg aliquots of total protein were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Hybond-C extra; Amersham). After blocking, the membrane was probed with a 1:100 dilution of anti-HLA-G mAb 4H8 (22) directed against the α1 domain of the HLA-G molecule (kindly provided by M. McMaster). Antibody binding was detected using a peroxidase-conjugated secondary antibody (Sigma) and chemiluminescence (ECL+ detection kit; Amersham).
RESULTS

Detection of HLA-G mRNA Levels in Human Melanoma Biopsies. HLA-G mRNA levels in 28 melanoma specimens (described in Table 1) were obtained from the primary or metastatic tumors of 23 melanoma patients as well as healthy skin biopsies from 18 of the 23 patients, and tested by RT-PCR. In healthy skin samples, RT-PCR resulted in detection of basal low levels of the most abundant HLA-G1 transcripts, whereas the HLA-G2 transcripts were rarely detected, a pattern that is comparable to that described in peripheral blood lymphocytes (Fig. 1B; Refs. 33, 34). In 12 of 18 matched healthy and melanoma tissues, we detected a higher level of HLA-G transcription in tumor tissue than in the same patient’s healthy tissue (Figs. 1 and 2 and Table 1). Six of the 23 patients exhibited the same basal low level of HLA-G transcription in both tumor and healthy skin biopsies. A high level of HLA-G transcription, comparable to that observed in trophoblast-derived JEG-3 cells, was observed in five melanoma patients (VIG, CHO, ESN, GOU, and ETI; Fig. 1). The HLA-G transcription level observed at various tumor sites in the same patient were comparable, transcription activation being detected in both lymph node and s.c. skin metastases. Representative cases are shown in Fig. 1, and the results of analysis in other patients are summarized in Table 1.

Differential Expression of Soluble HLA-G Transcripts in Melanoma Biopsies. Specific RT-PCR analysis of soluble HLA-G5 RNA transcripts, using primers that amplify only the full-length secreted isoform, allowed us to detect differential transcription of soluble HLA-G5 transcripts in several patients (Fig. 2). Among the five melanomas that exhibited high levels of transcription, three (ESN, VIG, and ETI) had low or undetectable HLA-G5 transcript levels, whereas the two remaining tumor specimens (CHO and GOU) exhibited overall enhancement of all HLA-G transcripts. The three melanoma biopsies (MEN, PER, and LOR) that had lower HLA-G mRNA levels were also found to express high levels of the soluble HLA-G transcript. Representative cases are shown in Fig. 2, and the results of analysis in other patients are summarized in Table 1.

Detection of HLA-G Proteins in Human Melanoma Biopsies. HLA-G protein expression in melanoma tissues was evaluated by immunofluorescence, using mAb 87G, which is specific for the full-length HLA-G1 isoform. A correlation between high levels of HLA-G transcription and the expression of HLA-G protein was found in six melanoma biopsies obtained from four (ETI, CHO, ESN, and VIG) of the five patients in whom high levels of HLA-G mRNA were detected. For patient ESN, it was possible to analyze healthy skin as well as skin and lymph node metastases. HLA-G expression was selectively detected in both tumor sites but not observed in healthy skin (Fig. 3A). Biopsies of patients ETI (Fig. 3A) and CHO (data not shown), in whom a high level of HLA-G transcript was detected, were also positively stained by the anti-HLA-G antibody. Six biopsies of healthy skin obtained from melanoma patients as well as one sample of healthy skin obtained from normal breast plastic surgery, in which low or intermediate levels of HLA-G transcripts were detectable, were negative with the HLA-G antibody. Primary skin tumors of patients MEN and PER and skin metastasis of patient DES, all of which exhibited low levels of HLA-G transcription, were also negative for HLA-G protein expression. One of these tumor specimens (FLI) exhibited only moderate levels of HLA-G transcripts, similar to those found in healthy skin, and low HLA-G expression was reproducibly observed in this tumor by immunofluorescence (data not shown), indicating that low HLA-G transcription can still generate cell surface HLA-G protein expression. No immunofluorescence staining was detected with 87G in the healthy skin of this patient.

Western blot analysis, using the anti-HLA-G 4H84 mAb, revealed the presence of a 60,000 band in protein extracts from lymph node metastatic tumor samples obtained from patients ESN, VIG, and ETI. This band is similar to one observed in the JEG-3 choriocarcinoma cell line, known to express the HLA-G1 protein. No signal corresponding to HLA-G was detected in healthy skin (Fig. 3B).

Levels of HLA-G Transcription and Protein Expression Measured in Healthy Skin, Skin Primary Tumor, Tumor Regression Site, and Melanoma Metastasis in the Same Patient Were Found to Correlate with the Proportion of Tumor Cells That Expressed Melanoma-associated Antigens. In one patient (VIG), it was possible to test healthy skin, primary cutaneous tumor, lymph node metastasis, and tumor regression within the skin primary tumor site specimen. Activation of HLA-G transcription was high in this patient’s primary and metastatic tumor specimens but low and comparable in both healthy skin and tumor regression sites (Fig. 4). No transcripts

Fig. 3. Confocal microscopy analysis of HLA-G expression in melanoma biopsies and Western blot detection of the HLA-G protein in melanoma protein extracts. A, 87G positive staining of a skin metastasis section from ESN (b), lymph node metastasis from ESN (c), and lymph node metastasis from ETI (d), whereas the healthy skin sample from ESN remains unstained with the 87G antibody (a), displaying good correlation with results obtained at the transcription level. B, the M r 39,000 protein, corresponding to the HLA-G1 isoform, detected by the anti-HLA-G 4H84 antibody in the JEG-3 choriocarcinoma cell line used as the positive control, and in lymph node metastasis protein lysates from VIG, ETI, and ESN. Healthy skin from MAL (HS) was tested as a negative control, and no M r 39,000 band was detected.

Fig. 4. RT-PCR analysis of HLA-G transcripts in biopsies from patient VIG. Analysis of HLA-G transcription in patient VIG: healthy skin (HS), skin primary tumor (SPT), lymph node metastasis (LNM), and tumor regression (R). HLA-G transcript levels were analyzed in the same patient, using pan-HLA-G or HLA-G5-specific primer sets.
corresponding to HLA-G5 were detected in these samples. HLA-G, melanoma antigen, and classical class I protein expression were analyzed in primary skin tumor, lymph node metastases, healthy skin, and a tumor regression site in this patient. Healthy skin from this patient was negative for 87G (Fig. 5A). Both the nodular area of primary melanoma and lymph node metastases exhibited strong homogeneous staining with anti-HLA-G, anti-classical HLA class I, and anti-melanoma-associated antigen antibodies (Fig. 5, B and C). No staining could be detected in a regressive area of the primary tumor in the same patient (Fig. 5D).

DISCUSSION

Constitutive and inducible HLA-G expression has been demonstrated in cultured tumor cell lines (19, 35, 36). HLA-G protein expression in surgically removed tumor biopsies has also been analyzed in a limited series of colorectal cancer, laryngeal carcinoma, kidney, thyroid, and breast cancer. Such expression remains controversial (36–38) and is probably underestimated because of the paucity of reagents able to detect HLA-G isoforms other than the full-length HLA-G1 membrane-bound isoform. The results obtained in this study are new findings demonstrating that primary and metastatic melanoma cells can express HLA-G in vivo. Various mechanisms can account for the escape of melanoma cells from immune destruction, including down-regulation of MHC class I molecules, FasL expression (39), and secretion of inhibitory factors such as interleukin 10 and transforming growth factor-β by tumor cells (40). Peptide-based vaccine and adoptive transfer clinical approaches do not always counterbalance the selection capacity of escape mechanisms provided by tumor cell phenotype heterogeneity. Apart from the commonly held opinion that tumors are poor stimulators of immune responses, recent evidence favors an active role of tumors in inducing T-cell tolerance (41).

Some phenotypical features, such as expression of the Fas ligand, are common to immune-privileged sites in which tolerance is established, such as trophoblast and the anterior chamber of the eye, where HLA-G expression is also physiologically restricted. We demonstrate here that ectopic HLA-G gene transcription and protein expression can be detected in melanoma cells ex vivo. Several genes, including MAGE, that exhibit testis- and placenta-restricted distribution are

Fig. 5. HLA class I, HLA-G and melanoma antigen expression in healthy skin, skin primary tumor, lymph node metastasis, and tumor regression tissues of patient VIG. A–D, immunohistochemical analysis of HLA-G expression in biopsies from patient VIG. The 87G antibody (c) binds only to skin primary tumor (b) and lymph node metastasis (c) sections that were stained with the HMB45 anti-melanoma-associated antigen antibody (d). Healthy skin (A) and tumor regression (D) biopsies were not stained by either 87G (c) or HMB45 (d) antibodies. Positive staining with the antibody W6/32 can be seen in the four specimens (b), whereas the negative control (a) remains unstained.
activated in melanoma. These genes are thought to be activated through demethylation processes or by release from stringent genetic control, which could also trigger the expression of other genes, including HLA-G (42). We have shown that activation of HLA-G gene transcription and protein expression can occur in melanoma cells and could be considered a way the immune system is rendered ineffective against HLA-G-positive tumor subsets. This could occur by inhibiting the cytotoxic function of immune effector cells (19) through interaction with KIRs of both NK and T cells. Recent works show that the expression of immunoglobulin superfamily KIR receptors that interact with HLA-G is not restricted to NK cells. In addition to NK cells, the ILT2 receptor is expressed in T-cell subsets, B lymphocytes, and myelomonocytic cells (27, 43) and ILT4 is selectively expressed in monocytes, macrophages, and dendritic cells (26). Other as yet unidentified immunological functions of HLA-G expression in melanoma, such as enhancement of HLA-E expression (28, 44) and interaction with other activating or inhibitory receptors expressed on various immunocompetent cells, may, thus, become evident in the near future.

The capacity of tumors to secrete the soluble HLA-G isoform could also favor their spread. Soluble HLA-G5, similar in structure to full-length HLA-G1, lacks the transmembrane domain and includes a specific protein sequence translated from “intronic” mRNA. The soluble form of the HLA-G protein binds the same endogenous peptide motifs as HLA-G1 but differs with respect to TAP association (32). The soluble HLA-G5 antigen is secreted by cytrophoblasts and activated placental macrophages (45) and is also detected in serum and amniotic fluid (46). However, to the best of our knowledge, no data are available concerning secretion of sHLA-G by malignant cells and its possible functional implications. The release of soluble molecules, such as CD59/ICAM-1 (47), GD3 gangliosides (48), or HLA antigens (49, 50) has been described as a factor favoring in vivo escape from constitutive or treatment-induced immune control. Soluble class I molecules have been shown to inhibit the NK cytotoxic effector function (51) and may regulate interactions with cellular and humoral immune responses in the host, thus contributing to and facilitating the establishment of tolerance to tumor cells. Soluble classical class I molecules that were shed from cell membranes have been detected in the serum of patients with autoimmune and inflammatory diseases and after allogeneic organ transplantation (52). Although the biological significance of soluble class I molecules remains enigmatic, their capacity to induce apoptosis in alloreactive cytotoxic lymphocytes has been demonstrated (53) to enable down-regulation of T-cell responses. The soluble HLA molecules that present self-peptides are also thought to play a role in maintaining self-tolerance. When expressed in tumors, HLA-G secretion could have such an inhibitory effect on T-cell responses (54). The observed discrepancy between membrane-bound and soluble HLA-G isoforms at the transcription level is reported here for the first time and could represent adaptive mechanisms of tumor clones enabling them to selectively express membrane-bound isoforms. Further studies are needed to investigate the secretion of HLA-G protein and its potential correlation with disease severity. Whether the absence of HLA-G secretion would lead to a stronger immune response and to a better prognosis is also an interesting question. The roles of HLA-G isoforms that lack one or two extracellular domains also remain to be elucidated in the context of tumor escape mechanisms. Considering the accumulation of recent data related to HLA-G function in the establishment of immune tolerance, it is tempting to postulate that HLA-G expression could favor the appearance of metastatic clones. Although the limited number of patients included in this study did not allow us to correlate HLA-G expression in tumor cells or the secretion of soluble HLA-G with the severity of disease or with metastatic potential, this work clearly establishes the fact that such HLA-G expression in melanoma is, indeed, observed in vivo. Additional follow-up studies of HLA-G expression status in primary tumors regarding their capacity to metastasize will permit evaluation of this parameter as a facilitator of tumor progression.

Analysis of the HLA-G status of tumors before and during treatment and the design and development of pharmacological tools that selectively down-regulate HLA-G expression at the surface of HLA-G-positive tumor cells should help to provide complementary therapeutic approaches in the treatment and follow-up of human melanoma.

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HLA-G EXPRESSION IN HUMAN MELANOMA BIOPSES


Heterogeneity of HLA-G Gene Transcription and Protein Expression in Malignant Melanoma Biopsies


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