Cell. Mol. Life Sci. (2011) 68:417–431
DOI 10.1007/s00018-010-0583-4

Cellular and Molecular Life Sciences

MULTI-AUTHOR REVIEW

Biology of HLA-G in cancer: a candidate molecule for therapeutic intervention?

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Received: 21 October 2010 / Accepted: 22 October 2010 / Published online: 10 November 2010

Abstract Although the expression of the non-classical HLA class I molecule HLA-G was first reported to be restricted to the fetal–maternal interface on the extravillous cytotrophoblasts, the distribution of HLA-G in normal tissues appears broader than originally described. HLA-G expression was found in embryonic tissues, in adult immune privileged organs, and in cells of the hematopoietic lineage. More interestingly, under pathophysiological conditions HLA-G antigens may be expressed on various types of malignant cells suggesting that HLA-G antigen expression is one strategy used by tumor cells to escape immune surveillance. In this article, we will focus on HLA-G expression in cancers of distinct histology and its association with the clinical course of diseases, on the underlying molecular mechanisms of impaired HLA-G expression, on the immune tolerant function of HLA-G in tumors, and on the use of membrane-bound and soluble HLA-G as a diagnostic or prognostic biomarker to identify tumors and to monitor disease stage, as well as on the use of HLA-G as a novel therapeutic target in cancer.

Keywords HLA-G · Tumors · Immune escape

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC	Antigen-presenting cells
ARE	AU-rich elements
β2-m	β2-microglobulin
BCC	Basal cell carcinoma
BM	Bone marrow
cHL	Classical Hodgkin lymphoma
CLL	Chronic lymphatic leukemia
CREB	Cyclin AMP-response element binding protein
COBRA	Combined bisulfite restriction analysis
CTL	Cyotoxic T lymphocytes
DAC	5-Aza-2-deoxycytidine
DC	Dendritic cells
DLBL	Diffuse large B cell lymphoma
EBV	Epstein Barr virus
FAB	French, American, British
GM-CSF	Granulocyte-macrophage colony stimulating factor
GVHD	Graft versus host disease
HDAC	Histone deacetylase
HIF	Hypoxia inducible factor
HPV	Human papilloma virus
HRE	Hypoxia response element

HRS	Hodgkin Reed Sternberg
IFN	Interferon
IHC	Immunohistochemistry
LCR	Locus control region
LIF	Leukemia-inhibitory factor
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
miRNA	MicroRNA
MM	Multiple myeloma
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-κB
NHL	Non-Hodgkin lymphoma
NK	Natural killer
RCC	Renal cell carcinoma
RREB1	Ras-responsive element binding protein
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial lesions
sHLA-G	Soluble HLA-G
SNP	Single nucleotide polymorphism
TGF-β	Transforming growth factor
TIL	Tumor-infiltrating lymphocytes
Treg	Regulatory T cell
UTR	Untranslated region

Characteristics of the HLA-G molecule

Structure and physiological expression of HLA-G

Despite its localization within the major histocompatibility complex (MHC) at chromosome 6p21.3, HLA-G has been termed as non-classical HLA antigen since it differs from classical HLA class I molecules by its genetic diversity, structure, expression and function. In contrast to the highly polymorphic HLA class I molecules HLA-G exhibits a limited polymorphism with 43 alleles, which is distributed within the α1, α2 and α3 domains. Under physiological conditions, the constitutive HLA-G expression is highly restricted to fetal tissues such as amniotic cells, erythroid precursors and cytotrophoblasts, and in adults to immune-privileged organs including cornea, thymus, pancreatic islets, endothelial cell precursors and erythroblasts, as reviewed in [1]. Other cells, such as dendritic cells (DC) and macrophages can also express HLA-G. Furthermore, HLA-G expression can be induced in different diseases including cancers, transplantation, multiple sclerosis, inflammatory diseases and viral infections.

HLA-G differs from other HLA class I molecules since it can generate seven alternative mRNAs encoding four membrane-bound (HLA-G1–HLA-G4) and three secreted isoforms (sHLA-G; HLA-G5–HLA-G7), which lack the transmembrane domains. In addition, HLA-G1 can also be released into the medium by proteolytic cleavage as shed HLA-G1 (sHLA-G1) [2]. The alternate splicing of primary transcripts represents a key feature of HLA-G, is strictly controlled, and may be cell type-dependent regulated. So far, most of the available information concerns the HLA-G1 molecule and its soluble counterpart HLA-G5. They are composed of the heavy chain (HC) consisting of three globular domains (α1, α2, α3) non-covalently bound to β2-microglobulin (β2-m) and a nonapeptide. In contrast, the other isoforms lacking one or two globular domains are smaller and can neither bind β2-m nor present peptides.

Functional characteristics of HLA-G

It has been well documented that HLA-G exhibits an immune tolerant function by inhibiting different immune-competent cells. This inhibitory effect is mediated by direct binding of both soluble and membrane-bound HLA-G to inhibitory receptors, such as the immunoglobulin-like transcript (ILT) receptor 2 (CD85j; LILRB1) present on lymphoid and myelo-monocytic cells and ILT-4 (CD85d; LILRB2) expressed by DC, macrophages and monocytes. In addition, the killer cell immunoglobulin-like receptor (KIR) 2DL4/p49 (CD158d) expressed by NK cells also represents an HLA-G-specific receptor. Thus, HLA-G can directly interact with different immune cell subpopulations including NK cells, T cells and professional antigen-presenting cells (APC), as well as endothelial cells via these receptors [3–6]. This interaction is accompanied by the generation and maintenance of tolerance at different stages of the immune response, e.g., differentiation, proliferation, cytolysis and cytokine secretion.

Since HLA-G antigens can be expressed on tumor-infiltrating immune cells (TIL) as well as in peripheral blood from cancer patients [7–9], HLA-G has been suggested to impair patients' immune response against their own tumor. Indeed, HLA-G$^+$ APC can inhibit the function of CD4$^+$ T cells and induce their differentiation into regulatory T cells (T reg) [10].

Next to these direct effects, HLA-G can exert its immune suppressive activity by indirect mechanisms, in particular by the expression of the non-classical HLA class I molecule HLA-E, which directly binds peptides derived from HLA-G. This molecule can interact with the inhibitory receptor CD94/NKG2A resulting in the inhibition of NK and T cell reactivity. In addition to this general effect of HLA-G on immune responses, different HLA-G isoforms may also exert distinct immune activities. For example, the soluble HLA-G5 protein can induce apoptosis of activated CD8$^+$ T and NK cells mediated by a FAS/FAS-L-dependent mechanism. In addition, HLA-G may exert its inhibitory activity through a feedback mechanism

due to the inhibition of the proliferative response of HLA-G5-secreting alloreactive $CD4^+$ T cells.

HLA-G expression in tumors of distinct histology

In this section, we describe HLA-G expression in tumor lesions. We will distinguish hematological malignancies, which include acute leukemia and lymphoproliferative disorders constituting a heterogeneous group of diseases, and solid tumors of distinct histology.

HLA-G expression in hematological malignancies

Acute leukemia

HLA-G has not been generally detected on acute leukemic cells [11, 12] with the exception of one study, in which HLA-G was expressed in 18.5% of acute myeloid leukemia (AML) cases [13]. HLA-G serum levels were increased in patients with AML, especially in subtypes affecting monocytic and lymphoid lineages such as FAB M4 and FAB M5 as well in patients with B and T acute lymphoid leukemia (ALL). Cytokine stimulation with GM-CSF and IFNγ increased HLA-G secretion in FAB M4 AML [14].

Lymphoma

Using immunohistochemistry of paraffin-embedded tissues, HLA-G protein was found in 23/45 cases of cutaneous lymphoma including 7/10 cutaneous B cell lymphomas and 16/35 cutaneous T cell lymphomas [8]. HLA-G expression was associated with IL-10 expression in 73% of the HLA-G^+ cases. Furthermore, Dummer and his collaborators showed that primary cutaneous $CD8^+$ and $CD56^+$ T lymphomas expressed HLA-G and the receptor ILT2 [15].

In chronic lymphatic leukaemia (CLL), HLA-G is expressed either as membrane-bound or as soluble form. In some studies, HLA-G surface expression has been reported to vary in leukemic cells between 1 and 54% [16–18], whereas one of us did not detect HLA-G on the cell surface, but reported a significant increase of plasmatic sHLA-G levels in 59–70% of CLL patients [19, 20]. In classical Hodgkin's lymphoma (cHL), HLA-G protein was expressed by Hodgkin Reed Sternberg (HRS) tumor cells in 54% of the cases (95/175); it was associated with lack of HLA class I cell surface antigen expression by HRS cells and with an Epstein Barr virus (EBV) negative status [21].

HLA-G cell surface expression was rarely detected in NHL using flow cytometry. Indeed, it was expressed on the cell surface in only 7 out of 110 B cell lymphoma cases analyzed. A partial HLA class I loss was reported in 3 out of 7 cases including two transformations of follicular NHL. None of the 52 low grade NHL expressed HLA-G protein in contrast to diffuse large B cell lymphoma (DLBCL) or high-grade lymphoma. Among the 7 DLBCL expressing HLA-G, 2 were de novo cases with EBV-induced NHL and 5 were transformations of quiescent lymphoma in DLBCL [22]. A significant increase of sHLA-G plasma levels was observed in 65% of B-NHL and 58% of T-NHL [20]. To assess the mechanisms involved in this secretion, the effect of cytokines was investigated, and a significant induction of HLA-G secretion was found by T lymphomatous cells stimulated, for example, by IFNγ, IL-2 or IL-10. This effect of cytokines was neither observed on B-NHL nor on healthy peripheral B or T lymphocytes and could be partially explained by the importance of the cytokine environment in these T cell lymphomas. However, in B-NHL, the role of the microenvironment recently appeared essential in the lymphomagenesis process. HLA-G expression in B-NHL could be explained by the presence of monocytes in the micro-milieu of B-NHL. In vitro studies observed an increase of HLA-G secretion after addition of monocytes to B lymphomatous cells, which was dependent on the monocytes/B lymphoma cell ratio [20]. The role of monocytes was in accordance with their physiologic expression of the ILT2 receptor. In addition, higher sHLA-G levels were found in Waldenstrom macroglobulinemia, in IgM MGUS, and in multiple myeloma (MM) than in healthy donors [23].

Allogeneic transplantations of hematopoietic stem cells is a curative therapy for several hematological malignancies, but the main complication is graft versus host disease (GVHD). HLA-G seems to be involved in the prevention of GVHD. High sHLA-G levels before and after allograft represent an indicator for favorite prognosis since increased HLA-G plasma concentrations were observed in patients without GVHD [24].

HLA-G expression in solid tumors

HLA-G was first shown to be expressed in melanoma in 1998 [36]. In the following years, HLA-G expression was frequently detected in solid tumors of distinct origin as summarized in Table 1. These include, for example, non-melanoma skin cancers, lung carcinoma, prostate adenocarcinoma, bladder cancer, gliomas, ovarian carcinomas, endometrial adenocarcinoma, breast cancer, gastric tumors, and colorectal cancers, as well as renal cell carcinoma (RCC).

What emerges from all these studies is that HLA-G could be potentially expressed by all tumor types. The level of expression varies between 0 and 100% for the different tumor types studied. No HLA-G expression was found in uveal melanoma and laryngeal carcinoma, whereas

Table 1 Frequency of HLA-G transcripts, mRNA isoforms, membrane-bound and soluble HLA-G isoforms in solid tumor lesions and/or tumor cell lines of distinct origin

Type of tumors	Protein	Elevated sHLA-G	mRNA	mRNA isoforms	Reference
Melanoma	30%	NT	70%		[73]
	26/84	NT	23/23	G1, G2, G3, G4, G5	[28, 29]
	9/24	NT	NT		[123]
	NT	++	NT		[103]
Non-melanoma skin cancers					
SCC	13/37	NT	NT		[124]
In situ carcinoma	7/15				
AK	6/22				
BCC	1/7				
Keratoacanthoma	1/5				
Renal cell carcinoma					
Clear cell carcinoma	7/12	NT	NT		[125]
Cell lines	6/14	NT	8/14	G1/G5 > G3 > G2/G4	[55]
Tumor specimens	12/18	NT	+		[126]
Bladder carcinoma	7/42		12/42		[50]
Prostatic adenocarcinoma	+	NT	+	HLA-G1/G2/G5/G6	[34]
Oesophageal squamous cell carcinoma	110/121	NT	NT		[53]
Gastric cancers	52/115	NT	NT		[127]
	113/160	NT	NT		[52]
Colorectal cancers	130/201	NT	NT		[49]
	21/39	NT	34/39		[128]
Breast cancer	14/36	NT	10/32		[30]
	16/39	NT	+		[129]
Endometrial adenocarcinoma	24/44	NT	+/−		[130]
Ovarian carcinoma	12/34	NT	NT		[33]
	45/74	59/60	+		[32]
	9/17	NT	NT		[43]
Endometrial adenocarcinoma	24/44	NT	7/10		[130]
Gliomas	4/12 cell lines		6/12		[26]
	4/5 brain tumors				
Lung malignancies	12/36	NT	NT		[7]
Non-small cell lung cancer	42/101	++	NT		[51]

AK Actinic keratosis, *SCC* squamous cell carcinoma, *BCC* basal cell carcinoma, *NT* not tested

hydatiform moles expressed HLA-G in 100% of cases due to their placental origin [25]. The frequency of HLA-G-expressing malignant cells present in a tumor does not appear to be essential for the induction of immune tolerance since in gliomas already 10% of HLA-G^+ tumor cells exerted significant immune inhibitory effects [26]. This finding might be due to trogocytosis as described later [27].

When comparing HLA-G expression between tumor tissues and corresponding adjacent tissues, the conclusions that can be drawn are as follows: in most cases, HLA-G is expressed by tumor cells and not by the surrounding healthy tissues. A sequential study in melanoma illustrates this point, demonstrating that HLA-G protein was expressed in both primary and metastatic lesions, but was not detected in healthy skin or during tumor regression [28].

HLA-G may be expressed by non-tumor cells, like tumor-infiltrating mononuclear cells, independently of HLA-G positivity of tumor cells. For example, in breast, lung and ovarian carcinoma as well as in melanoma, HLA-G was detected either in the infiltrating immune cells or in tumor cells depending on the patients analyzed [29, 30]. Lefebvre et al. [30] found HLA-G expression on tumor epithelial cells and on immune cells such as macrophages and $CD8^+$ T lymphocytes in 14 out of 36 breast cancer lesions characterized by a high infiltration with inflammatory immune cells. Furthermore, in ovarian cancers, which HLA-G is

detected in more than 50% of cases [31, 32], HLA-G was also found on both tumor and tumor-infiltrating immune cells [33]. In contrast, in malignant lung diseases, HLA-G expression was detected on activated macrophages and DC, but not on tumor cells, in approximately 30% of the cases analyzed [7].

More rarely, the non-tumor cells expressing HLA-G can be detected in the surrounding non-tumoral tissue as was recently shown for the HLA-G5 protein in normal prostate and in prostatic secretions [34]. This might be explained by the inflammatory microenvironment within tumor lesions that could induce HLA-G expression. This hypothesis was supported by a study using osteoarthritis as a model demonstrating that inflammatory stimuli such as IL-10 induce sHLA-G production [35]. Thus, HLA-G expression by tumor cells and/or other non-tumor cells might be the consequence of distinct mechanisms, may have different pathological significance, and either a deleterious or beneficial effect, which will be addressed in "Functional relevance of HLA-G in tumorigenesis: HLA-G and immunoediting".

Discrepancies about HLA-G expression have been reported in some cancers

There exists abundant literature about HLA-G in tumors, which was published during two distinct periods. During the years 1998 and 2002, 27 articles reported HLA-G expression in tumors, whereas 11 reported a lack of HLA-G expression even for the same tumor entity. For example, the initial expression of HLA-G in tumors was described in melanoma by Paul et al. [36], but Frumento et al. [37] detected no HLA-G expression in melanoma lesions and in melanoma cell lines after induction by IFNγ. In acute leukemia stimulation with IFNγ induced HLA-G expression on AML cells according to Mizuno et al. [38], whereas Fauchet and her collaborators [11] described HLA-G induction only on U937, a monohistiocytic cell line, but not in leukemic cells. However, despite the controversial results at that time, the discrepancies observed in the first period can now be explained by technical reasons involving reagents and methods. At the beginning of the HLA-G studies, no commercial antibodies were available and the anti-HLA-G reference antibodies 87G and 4H84 were kind gifts of D. Geraghty and Mc Master, respectively. The HLA-G, -E, -F pre-workshop validated reagents and protocols for the analysis of HLA-G expression [39]. With the commercialization of monoclonal antibodies (mAb) such as MEM-G/− antibodies, 87G, 4H84 and G233, their characterization for use in flow cytometry, western blotting and immunochemistry [40] and the international collaborative works, these mAbs have been validated as reliable reagents for HLA-G analyses. Subsequently, the number of publications after 2002 reporting a lack of HLA-G expression dropped to 1, whereas additional 47 papers described the expression of HLA-G in tumors.

Concerning the methodology, the use of a correct isotypic control is important for validating small percentages of positivity. In some papers, an IgG1 antibody was used as istotype control for 87G instead of IgG2a that could explain some differences in the results since the background is higher for the IgG2a subtype. Furthermore, the percentage of human AB serum used to block Fc receptors of immunoglobulins also appears to be important in particular by analyzing cells from the monocytic lineage [11, 38]. Finally, the methods employed for analysis could be an additional explanation: flow cytometry is more sensitive than immunohistochemistry, and multicolor flow cytometry still allows sensitivity gain over simple or double color labeling.

In addition, there exists an obvious discrepancy between the expression of HLA-G in surgically removed tumor lesions and in established long-term tumor cell lines. Indeed, initial studies focused on cell lines and did not detect any HLA-G protein expression [41]. This was due to the gradual loss of HLA-G by tumor cells cultured in vitro as shown in melanoma [42], ovarian carcinoma [43] and RCC [44]. It could be explained by (1) the heterogeneity of the tumor cell populations and (2) certain micro-environmental factors necessary for HLA-G induction and/or maintenance.

Functional relevance of HLA-G expression in cancers

In this section, we will first review the cancers in which a correlation has been found between HLA-G expression and clinical/biological parameters. Then, the functional relevance of HLA-G in tumorigenesis according to the three steps of cancer immunoediting will be addressed [45].

Correlation between HLA-G expression and clinical/biological parameters

In CLL, a significant correlation was found between HLA-G positivity (>12%) and progression-free survival of patients [46]. A correlation with hypogammaglobulinemia that is representative of immunosuppressive status was described by some authors [20], which was also associated with a reduced treatment-free survival for another group [17]. In acute leukemia, HLA-G expression in 18.5% cases of AML was correlated with unfavorable clinical implications [47], whereas an another team described a link between elevated sHLA-G plasma levels and the absence of anterior myelodysplasia as well as high leukocytosis using ANOVA criteria [14].

In basal cell carcinoma (BCC), HLA-G protein was expressed at a higher frequency in aggressive compared to non-aggressive superficial and nodular BCC [48]. In colorectal cancers, HLA-G protein expression was detected in 130/201 (64.6%) of primary lesions and served as an independent prognostic factor: patients with HLA-G^+ tumors exhibited a significantly shorter survival time [49]. Concerning bladder cancer, there was a significant increase in HLA-G expression in cases with metastatic prostate infiltration [50]. In lung carcinomas, membrane-bound HLA-G expression was found in 41.6% of primary non-small cell lung cancer lesions and plasma sHLA-G levels were related to the disease stage [51].

HLA-G protein expression was observed in 71% of primary gastric carcinomas and its expression was significantly correlated with the histological grade and clinical stages of disease. Furthermore, patients with HLA-G^+ tumors had a significantly shorter survival time [52]. Expression of HLA-G was also found in 90.9% of esophageal squamous cell carcinoma (110/121) and was significantly associated with a poor patient prognosis [53]. A similar correlation was found in patients with advanced ovarian cancer exhibiting significant high levels of HLA-G mRNA and protein when compared to normal or early stage ovarian cancer lesions, which was associated with a poor patient outcome [54]. In addition, sHLA-G levels were significantly higher in malignant ascites than in benign controls and could therefore constitute a potential tumor marker in malignant ascites [32].

Functional relevance of HLA-G in tumorigenesis: HLA-G and immunoediting

The immune surveillance can be divided into three distinct steps of cancer immunoediting, in which HLA-G could also be involved [45]. The first step is represented by the elimination phase and involves the elimination of tumor cells by cells of the adaptive and innate immune systems. During this phase, local Th1 cytokines, such as IFN-γ, were produced by tumor-infiltrating lymphocytes (TIL) and NK cells. This IFN-γ-mediated up-regulation of HLA-G expression has been shown in glioma and RCC cell lines at the mRNA and protein level [26, 55], thereby affecting the innate and adaptive immunity. Indeed, HLA-G could inhibit the function of immune effector cells and APC by binding to its receptors ILT-2, ILT-4, and KIR2DL4/p49, thereby blocking the cytolytic function of T lymphocytes and NK cells [56, 57] via ILT2 and their proliferation via ILT2 and ILT4 [58, 59], as well as inducing apoptosis via CD8 [60]. HLA-G could also affect the maturation, migration, trafficking, antigen presentation [61] and the cross-talk of DC with T and NK cells [62].

In vitro studies have shown that HLA-G-transfected melanoma, glioma, ovarian, renal, and lung carcinoma cell lines are protected from lysis by NK cells and/or antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL) [26, 43, 51, 63, 64]. HLA-G protein isoforms other than HLA-G1 protected melanoma cells from NK lysis [36]. This resistance to NK-mediated killing was also demonstrated using ex vivo isolated HLA-G^+ CLL and MM cells [65].

As expected, soluble HLA-G (sHLA-G) molecules have similar functional properties. It was shown that the HLA-G5 isoform secreted by M8 melanoma cells inhibited NK cell cytotoxicity by impairing lytic granules polarization toward the target cell [66]. In addition, plasmatic sHLA-G molecules observed in lymphoproliferative disorders were functional and strongly inhibited T cell proliferation at concentrations currently observed in these diseases [20].

Moreover, HLA-G is able to induce tolerogenic DC [67], and also suppressive NK or T cells, especially by trogocytosis [68, 69], which further strengthens the functional relevance of HLA-G expression. Trogocytosis is a mechanism of antigen exchange between interacting cells involving active, rapid intercellular transfer of membrane patches. It can occur between tumor cells and immune cells or between different types of immune cells including APC [27]. Indeed, HLA-G could be transferred from one HLA-G-expressing cell to other cells. Thus, this phenomenon could be a mechanism of immune inhibition and protection for HLA-G^- tumor cells that are in the vicinity of HLA-G-positive cells [1].

The second step of immune surveillance is defined by the equilibrium phase allowing cancer cell persistence, thereby resulting in the selection of tumoral clones with reduced immunogenicity. This phase is characterized by epigenetic changes of the HLA-G promoter [70], such as demethylation and histone acetylation leading to transcriptional activation of HLA-G.

In the last phase, called the escape phase, tumor development occurs. This might be at least in part due to mechanisms that allow immune escape such as, e.g., chronic inflammation associated with the activation of NF-κB, which increases proteolytic shedding leading to the production of HLA-G molecules [71], hypoxia and cytokine production. Indeed, the tumor-derived cytokines IL-10 and TGFβ, as well as hypoxia, could induce HLA-G expression. Altogethe,r these processes lead to tumor promotion by facilitating escape of tumor cells from tumor antigen-specific immune response.

In conclusion, the functional relevance of HLA-G varies according to its expression by tumor cells or tumor-infiltrating cells. When expressed by tumor cells, it constitutes an efficient way for escaping from immunosurveillance in view of its immunosuppressive properties. In contrast, HLA-G expression could be beneficial when it is expressed by tumor-infiltrating cells which bear ILT2, an inhibitory receptor known to interact with HLA-G [30]. Therefore, its

effect on immune cells could also consist of limiting their Th1 cytokines secretion and subsequently chronic inflammation associated with tumor growth.

Multiple HLA-G isoforms and their clinical relevance in cancers

The clinical relevance of HLA-G has been shown for both membrane-bound and soluble forms in several types of cancers (see "Functional relevance of HLA-G expression in cancers") such as melanoma and glioma. In this section, we address the characteristics of the HLA-G isoforms and their potential as tools.

The HLA-G structure is complex due to the alternative splicing of its gene, the powerful mechanisms of transcriptional and post-transcriptional regulation, the multiplicity of structures such as β2-m associated or not, monomers or multimers (see "Characteristics of the HLA-G molecule"). Furthermore, the type of structure adopted by HLA-G could influence its binding to ILT receptors [72].

HLA-G mRNA expression was detected in tumor tissues as well as in normal tissues, but the level of transcription was higher in tumors and the number of HLA-G isoforms strongly varied as shown for melanoma [36] and RCC [44]. The level of HLA-G transcription was comparable in the various tumor sites of the same patient [28]. All HLA-G mRNA isoforms could be detected in the IGR melanoma cell line and in melanoma biopsies in contrast to healthy skin, which exhibited only signals for HLA-G1 and/or -G5 [36].

Furthermore, HLA-G protein was found in tumors with high levels of HLA-G transcripts in melanoma [28]. However, the presence of HLA-G transcripts is not always equivalent to protein translation. A frequent transcription and a relatively rare translation is a combination observed in many tumors, as in RCC and in particular in lymphoproliferative disorders. This can be explained by powerful mechanisms of post-transcriptional regulation [55]. Stress factors, especially those present in the tumor environment, i.e. hypoxia, may participate in these regulatory mechanisms [73]. Thus, micro-environmental factors cannot only affect the transcriptional status but also the splicing. Indeed, a switch of alternative splicing leading to the loss of cell surface HLA-G1 and its replacement by intra-cellular HLA-G2 was observed that abrogates the functional role of HLA-G in tumor escape, since tumor cells following this switch became susceptible to NK cell-mediated lysis [42]. The expression of HLA-G truncated isoforms is potentially relevant in vivo because forms other than HLA-G1 can also inhibit NK cell-mediated cytolysis [74]. So far, no specific antibody is available to detect these isoforms. Therefore, it is postulated that the physiologic relevance of HLA-G as protein isoforms is under-evaluated.

Regarding the sHLA-G isoforms, sHLA-G detected by ELISA represents sHLA-G1 and HLA-G5, which correspond to the full length and complete isoforms. Antibodies available are MEM-G/9 recognizing both HLA-G5 and sHLA-G1 and 5A6G7 specific for the intron 4-retaining isoforms HLA-G5 and HLA-G6. In contrast, no commercial antibodies are specific for the HLA-G6 and HLA-G7 isoforms. As a result, the sHLA-G level might be underestimated in tumor lesions.

The discordant HLA-G mRNA and protein expression in lymphoproliferative disorders led us to investigate HLA-G levels in plasma of patients [22]. As expected, increased sHLA-G levels were found in the majority of the cases. In addition, an increase in HLA-G1 proteolyic shedding controlled by NF-κB inducers was described in tumor cells. On the other hand, matrix metalloproteinase (MMP) expression by tumors was increased in cancers, in particular MMP-9 in NHL. However, sHLA-G levels were comprised of both sHLA-G1 and HLA-G5 as shown in B lymphoma with HLA-G5 as the major isoform [20].

It has been further demonstrated that HLA-G can associate with β2-m or exist as free heavy chains. A novel quaternary structure of HLA-G5 was described for the first time in the placental villous cytotrophoblast cells, as β2-m-free, disulfide-linked HLA-G5H or H homodimers [75]. The authors explain the absence of the light chain by the low transcription of β2-m and by the inability to translate mRNA into protein. The lack of β2-m protein has been well described as a mechanism causing HLA class I deficiency in tumors [76, 77]. The existence of β2-m disulfide-linked HLA-G5 homodimers can therefore be postulated in cancers even if they have not yet been identified. It remains to be determined whether these homodimers could exhibit the same affinity for ILT receptors as the complete forms.

It is noteworthy that HLA-G can be found in different conformations at the cell surface and forms homomultimers of conventional β2-m-associated HLA-G complexes through the generation of Cys42-Cyst42 or Cys42-Cys147 disulfide bonds [78, 79]. HLA-G dimers bind ILT2 receptors with a higher affinity and slower dissociation rate than monomers [80]. HLA-G1 homodimers have been shown to be expressed at the surface of normal trophoblast in vitro [79] and of human extravillous trophoblast cells in vivo [81]. HLA-G5 homodimers have also been described in vitro [81]. β2-m-free HLA-G1 and -G5 can be detected on the cell surface or in culture supernatants of HLA-G-expressing cells [75]. Until now, no HLA-G multimers have been described in tumors, since the antibodies available cannot discriminate between these structures. The lack of tools to detect certain structures of HLA-G prevent the detection and/or titration of all HLA-G forms. Thus, the pathologic relevance of HLA-G might be underestimated in cancers.

Hence, there is a urgent need to precisely identify relevant HLA-G structures in vivo, in order to design better diagnosis and therapeutic strategies.

HLA-G as a diagnostic and prognostic tool

The three sHLA-G isoforms, HLA-G5 to -G7 are generated by alternative splicing, whereas sHLA-G can also be generated by proteolytic release of the HLA-G membrane-bound forms [1]. Analogous to classical HLA class I antigens, sHLA-G antigens are found in serum using a number of antibody-based assays. Serum HLA-G antigens are derived from the release of the membrane-bound HLA-G isoforms, such as HLA-G1, and from the secretion of sHLA-G isoforms, such as HLA-G5. The source of sHLA-G is represented by both immune cells and tumor cells. Indeed, not only monocytes, but also T cells and DC, secrete sHLA-G molecules in vitro. Furthermore, sHLA-G plasma levels are often significantly increased in patients with malignant diseases including melanoma, glioma, breast and ovarian carcinoma as well as in some lymphoproliferative disorders [17]. Serum sHLA-G was significantly elevated in melanoma patients compared to healthy controls. Since IFN treatment is used in patients with melanoma, the effect of IFN treatment on HLA-G expression was investigated in melanoma. Different studies showed controversial results: immunotherapy with IFNα-2b increased HLA-G surface expression as demonstrated by immunohistochemistry of paraffin-embedded tissue sections [5]. In addition, the level of sHLA-G was also elevated in melanoma patients and is increased by IFN-α immunotherapy [6].

In Waldenstrom macroglobulinemia and in IgM MGUS, comparable sHLA-G levels were found, which were higher than in healthy donors but have no significant clinical relevance. In contrast, soluble HLA class I antigens (sHLA-I) appeared to be correlated with known markers of poor prognosis, such as β2-m, in these diseases [52]. In MM and MGUS, median sHLA-G was significantly higher than in healthy controls, but only high levels of sHLA-I were predictive for short patient survival [53]. Concerning solid tumors, there exists a correlation between the sHLA-G serum levels, advanced disease stage, tumor load and the clinical outcome of patients. Higher serum sHLA-G levels were found in ovarian and breast tumor patients compared to healthy volunteers. In some tumors, this increase was associated with an unfavorable prognosis and reduced patients' overall survival [33, 82–85]. In this context, it is noteworthy that transplanted patients, who had increased sHLA-G serum levels, had an improved allograft acceptance. These data suggest that increased sHLA-G levels in biological fluids are associated with the down-regulation of immune responses.

Soluble HLA-G molecules were found to be increased in acute leukemia, in particular in subtypes affecting monocytic and lymphoid lineages, such as FAB M4 and FAB M5 AML as well as in B and T ALL. Incubation with cytokines such as GM-CSF and IFN-γ enhanced HLA-G secretion in FAB M4 AML and ALL. A link between elevated sHLA-G plasma levels and the absence of anterior myelodysplasia and high levels of leukocytosis was suggested using ANOVA criteria [37]. A significant increase of sHLA-G levels was found in 65% of B-NHL and 58% of T-NHL [45]. Thus, in some malignant diseases, HLA-G surface antigens and sHLA-G levels did correlate with a poor clinical outcome of patients.

Based on these results, the determination of sHLA-G levels might be used as a diagnostic tool to distinguish between malignant and benign tumors. In addition, HLA-G might serve as a possible marker for tumor sensitivity to chemotherapy and as a prognostic marker for advanced disease stage and clinical outcome. However, to strengthen the implementation of HLA-G as biomarker, HLA-G expression and serum levels have to be determined in a large series of patient samples. In addition, it has still to be analyzed whether HLA-G surface expression in tumor lesions is correlated with sHLA-G serum plasma levels. So far, either HLA-G expression in tumor lesions or sHLA-G in serum was determined, but only very few studies have analyzed both HLA-G and sHLA-G plasma expression in parallel. In this context, it is noteworthy that the frequency of HLA-G expression in tumors determined by IHC is lower than the antibody-based ELISA used to measure HLA-G in serum.

Molecular mechanisms involved in impaired HLA-G expression

Multiple molecular mechanisms have been suggested to play a role in the aberrant appearance of HLA-G antigens upon viral and malignant transformation. Since one major hallmark of cancer is its genetic instability associated with loss of organized genetic control in aggressive lesions, altered genomic control might be responsible for HLA-G re-expression in tumors. Furthermore, the presence of stable HLA-G transcripts is often, but not always, correlated with HLA-G protein expression, suggesting that HLA-G expression is regulated at the transcriptional, post-transcriptional and/or epigenetic level. In addition, HLA-G polymorphisms appear to be associated with distinct HLA-G expression levels. The characterization of the molecular mechanisms, which regulate the expression of this molecule in physiological and pathological conditions, will contribute to our understanding of the functional aspects of this protein. Furthermore, given the potential role of HLA-G in the clinical course of malignant diseases, the information about the processes regulating HLA-G may

contribute to the development of strategies modulating its expression and to optimizing the design of (immuno-) therapeutic strategies for the treatment of tumor patients. In this section, the distinct molecular mechanisms leading to altered HLA-G expression in tumors will be addressed by focusing in particular on the involvement of the tumor microenvironment, since HLA-G expression might be altered by changes in the micro-milieu associated with inflammation and hypoxia. A better understanding of the molecular mechanism(s) influencing HLA-G expression might lead to the development of strategies to manage the pathophysiologic conditions, in which HLA-G expression is involved, including pregnancy complications, organ transplantation and cancer.

Immune suppressive tumor microenvironment

Tumors often exhibit an immune suppressive micro-environment, which is not only characterized by the secretion of immune suppressive cytokines, such as IL-10 and the transforming growth factor (TGF)-β and metabolic products like lactate and nitric oxide [86], but also by the presence of immune suppressive cells like regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC). Inflammatory processes and neoplastic transformation further induce HLA-G in infiltrating macrophages and DC [87]. The time point of HLA-G activation and up-regulation in tumor cells remains unsolved since inflammation may promote tumor development. HLA-G up-regulation during chronic inflammation in addition to an increased growth of neoplastic cells will lead to escape of these cells from immune surveillance of the host.

Transcriptional regulation of HLA-G

HLA-G promoter structure

A major feature of HLA-G is the alternative splicing of the primary transcript thereby deleting specific exons or retaining introns, which results in the generation of seven distinct HLA-G isoforms, the membrane bound HLA-G1 to -G4 and the secreted HLA-G5 to -G7. Tumors exhibit a distinct pattern of HLA-G transcripts and protein isoforms. The diversity of the isoforms detected appears to be due to malignant transformation and the cellular micro-environment [88], although the role of this heterogeneity has not yet been determined [44]. In comparison to classical HLA class I genes, the 5′ upstream regulatory region of HLA-G includes not only the conventional (CCAAT and TATA box) elements for basal regulation and transcriptional initiation but also unique promoter sequences like a second putative initiation site of the HLA-G transcription, enhancer A, IFN-stimulated response element and the SXY sequence. With the exception of S1 and X1 boxes, classical HLA class I acting regulatory elements are disrupted within the HLA-G promoter [89, 90]. Due to these differences, the regulation of the HLA-G promoter activity is both quantitatively and qualitatively distinct from that of the gene products of other HLA class I loci. The HLA-G promoter binding factors and respective target sequences include three CRE/TRE elements mediating its regulation by the cAMP response element binding protein 1 (CREB1), the interferon regulatory factor (IRF)1, the heat shock transcription factor (HSF)1, the RAS response element binding protein 1 (RREB-1), and a novel progesterone response element [91–97]. Based on the transcription factor-binding sites, HLA-G expression is in part regulated at the transcriptional level. However, HLA-G is particularly unresponsive to NF-κB, IRF1 and the class II transactivator CIITA [98]. The constitutive activation of NF-κB is associated with chronic inflammation, which is often observed in tumors leading to tumor growth. Moreover, NF-κB activation affects HLA-G protein expression but not HLA-G transcription: it reduces HLA-G cell surface expression by increasing its proteolytic shedding in tumor cells [71].

Modulation of HLA-G transcription by different factors

Similar to classical HLA class I antigens, HLA-G expression can be unregulated at the mRNA and protein level by interferon (IFN)-γ, IL-10 and the leukemia inhibitory factor (LIF) [8, 99, 100]. The immunosuppressive cytokine IL-10 can be produced by tumor cells as well as by tumor-infiltrating cells. Interestingly, an association between HLA-G and IL-10 expression was found in some tumor entities. For example, in cutaneous lymphoma and lung cancer, HLA-G immunoreactivity was often associated with IL-10 expression [8, 48]. This HLA-G expression can in turn favor induction of a Th2 cytokine profile including IL-10 production. The consequence is the establishment of a vicious circle of immune suppression in cancers [101].

Immunotherapy using IFNs in certain cancers like melanoma and RCC aim at increasing tumor immunogenicity. However, this may lead to an induction of membrane-bound and soluble HLA-G expression as shown in melanoma patients treated with IFNα-2b [9]. The up-regulation of HLA-G in these tumors is associated with disease progression and a poor clinical outcome of patients [102]. This effect has to be taken more into account since HLA-G$^+$ melanoma cells secrete exosomes that contain HLA-G [103].

In addition, HLA-G expression can be enhanced by progesterone and completely inhibited by co-incubation with the progesterone antagonist RU486 [97]. Furthermore, progesterone can also induce HLA-G expression in

vascular endothelial and smooth muscle cells in vitro. These results suggest that changes of HLA-G expression in mammary carcinoma cells may be modulated by hormone treatment and therefore may affect the host immune status as well as the CTL response [93]. This possibility is supported by the association between HLA-G expression and the estrogen as well as progesterone receptor status in breast cancer. HLA-G down-regulation in the presence of progesterone antagonists might be an additional molecular mechanism, by which this substance improves the host's immune status.

Hypoxia is associated with rapid tumor growth and with metastases [104]. This is associated with the over-expression of the hypoxia-inducible factor (HIF1) in human primary cancers and metastases. The tumor-related stress induces HIF1-dependent HLA-G transcription in tumor cells. The HLA-G gene response to hypoxia is cell type-dependent, since in HLA-G- melanoma cells HIF1 could induce HLA-G expression [73], whereas an HIF-1-dependent decrease in HLA-G gene activity was detected in tumor cells constitutively expressing HLA-G [105].

Silencing of HLA-G expression mediated by epigenetic mechanisms

Although some tumors frequently express high levels of HLA-G transcripts and/or protein, others totally lack HLA-G expression. Depending on the cell type, different mechanisms are responsible for HLA-G silencing. These include epigenetic mechanisms, like CpG methylation and histone deacetylation [70, 106], or not yet identified factors, which negatively interfere with HLA-G expression [96]. So far, HLA-G methylation has been found not only in solid tumors, such as lung, colon, ovarian carcinoma and RCC, but also in hematopoietic malignancies [55, 70, 106–108]. The level of HLA-G promoter methylation significantly differed in the tumor cells analyzed, as demonstrated by methylation-specific PCR, bisulfite sequencing as well as by combined bisulfite restriction analysis (COBRA). Treatment of HLA-G- tumor cells with 5-aza-2-desoxycytidine (DAC) reversed HLA-G repression by demethylation of the HLA-G promoter, which enhanced HLA-G transcription and protein expression in some leukemia and solid tumor cells [70, 108], but only rarely induced HLA-G [55, 109]. In normal non-malignant tissues and cell lines, HLA-G methylation has either not been demonstrated or only detected at a low frequency [55, 107]. Using the HLA-G^+ and HLA-G- trophoblast-derived choriocarcinoma cell lines JEG3 and JAR as model systems, a similar extent of HLA-G methylation was found in both cell lines. On the other hand, the histone acetylation of the HLA-G promoter chromatin was enhanced in JEG3 cells compared to that of JAR cells, which is directly associated with the HLA-G expression levels. These data suggest that HLA-G expression is also regulated by histone modifications [110]. Thus, demethylating and histone acetylating agents used in therapies should be carefully evaluated due to their effect on HLA-G expression.

Association of HLA-G sequence polymorphism with its expression level

Unlike the polymorphism in classical HLA class I molecules, which are mainly concentrated around the peptide-binding groove, the limited HLA-G polymorphism is distributed between the α1, α2 and α3 domains (Donati et al., this special issue). So far, single nucleotide polymorphisms (SNP) have been described in the promoter region (SNP-964G/A), in exon 2 and exon 3 as well as in the 3′ untranslated region (UTR) of exon 8 of the HLA-G gene.

Besides the well-characterized polymorphisms at the promoter and in exons 2–4 of the HLA-G gene, a 14-base pair (bp) insertion/deletion polymorphism (SNP 3172G/A) as well as SNPs at positions 2,959, 2,995, 3,127, 3,142, and 3,181) have been described in the 3′-UTR (exon 8) [111–114], which appears in about 50% of caucasians suggesting a selective pressure on the deleted 14-bp allele. A high linkage disequilibrium among the variation sites was detected for the 14-bp insertion and the alleles +3142G and +3187A, although significant functional properties have only been associated with the 14-bp polymorphism SNP3172, SNP3142 and SNP3187A. Despite the requirement for systematic mutagenesis and transfection studies to clarify the role of the identified SNPs in the HLA-G gene in relation to its expression, the 14-bp polymorphism has been shown to account for differences in HLA-G mRNA and protein expression profiles. For example, lower HLA-G transcript levels were found in HLA-G genotypes with a 14-bp deletion, whereas lower levels of sHLA-G in serum and plasma, and differences in the alternative splicing of HLA-G transcripts and in HLA-G mRNA stability, were detected in +14/+14-bp HLA-G genotypes [47, 113, 115, 116].

These SNPs might be associated with certain diseases, such as autoimmune diseases, preeclampsia, transplantation and neoplasia [113, 117], and also with the clinical course of disease. So far, limited information has been published on the association of HLA-G polymorphism in tumor cells with the level of HLA-G expression and/or clinical outcome of patients. Recently, a study determined the HLA-G polymorphism in squamous intraepithelial lesions (SIL) harboring the human papilloma virus (HPV). A significant protective association was found between the presence of the G(*)0103 allele and SIL and between the G0101/G0104 genotype in high grade SIL. The +14 and

−14-bp haplotype conferred susceptibility to SIL, whereas the +14-bp haplotype in $HPV16^+/HPV18^+$ tumors is associated with high-grade lesions [118]. In addition, preliminary studies demonstrated a high frequency of this 14-bp polymorphism in RCC cells, which appears to correlate with the level of HLA-G expression (Seliger et al., unpublished results).

Post-translational control of HLA-G

Although HLA-G expression is mainly controlled at the transcriptional level, a post-transcriptional regulation of this gene has also been reported in a number of solid tumors of distinct origin, e.g., melanoma and RCC [28, 44]. The underlying molecular mechanism could be alternative splicing, altered mRNA stability and translation as well as impaired protein transport to the cell surface. In addition, it has been suggested that the discordant HLA-G mRNA and protein expression in human tumors might be mediated by HLA-G-specific microRNAs. MiRNAs are a family of endogenous small, non-coding RNAs of approximately 22 nucleotides, which regulate gene expression by suppressing translation or degrading mRNA [119]. These miRNAs are involved in many physiological and pathophysiological cellular functions. Using bioinformatic tools, a number of miRNA targets were predicted in the 3′-UTR of the HLA-G gene [111, 120]. Recently, the miR152 has been shown to reduce HLA-G expression, which is associated with an increased NK cell-mediated lysis [85]. This was further strengthened by miRNA arrays of tumor and corresponding non-malignant tissues, which identified putative HLA-G-specific miRNAs (Seliger et al., unpublished results), but their functional activity has still to be shown. Furthermore, the 3′-UTR polymorphism of the HLA-G gene can affect miRNA targeting suggesting that non-coding polymorphisms contribute to the risk of diseases [117]. Despite the evidence that miRNAs participate in the regulation of HLA-G expression in tumors, there might exist more functional HLA-G-specific miRNAs, which might be utilized to modulate HLA-G expression in tumors, thereby reverting their immune escape phenotype.

In addition to the postulated miRNA-mediated post-transcriptional control of HLA-G, the HLA-G protein can be nitrated [86]. The nitration of HLA-G depends on the protein concentration, the proximity to the site of generation of nitrating agents, and the tyrosine environment [121]. So far, the nitration of HLA-G has been shown in all tumor cells analyzed. However, this post-transcriptional modification does not interfere with the immune suppressive properties of HLA-G. Recently, an altered HLA-G transport has been shown, since HLA-G could recycle between the ER and the cis-Golgi until high affinity peptides are bound. This underscores the role of the ER, and also of the post-ER compartments in the quality control of HLA-G molecules, and further strengthens its translational regulation.

Modulation of HLA-G expression by the tumor microenvironment

There exists increasing evidence that interactions between genes and the environment play an important role in the etiology of common diseases including cancer. Changes in the tumor micro-environment may participate in the regulation of HLA-G expression. These tumor-induced alterations include stress and danger conditions, such as cytokines, heat shock, hormones (e.g., glucocorticoids) and hypoxia. However, for most of them, the molecular mechanisms remain to be elucidated. In tumors, the microenvironment has been shown to play an important role for the generation of an immune suppressive status mediated by tumor-infiltrating immune cells as well as by the induction of immune suppressive molecules, such as HLA-G [105].

HLA-G expression by tumor cells is associated with their reduced susceptibility to CTL- and NK cell-mediated lysis [122]. In RCC, normal levels of HLA class I surface expression is often accompanied by high levels of HLA-G expression, suggesting that tumor cells could evade recognition by HLA class I antigen-restricted, tumor antigen-specific CTL despite high levels of classical HLA class I antigens.

Functional significance of HLA-G expression in tumors

It is accepted that tumors often exhibit HLA class I abnormalities, which are often associated with the clinical course of disease and a limited patient survival. However, HLA class I-tumor cells may be killed by NK cells. Based on this assumption, it is suggested that malignant cells are not only protected from T cell- but also NK cell-mediated lysis, which might be due to an over-expression of HLA-G antigens. Indeed, in vitro studies in glioma, melanoma, and RCC cell lines demonstrated that, upon expression of endogenous HLA-G antigens, tumor cells were protected from lysis by alloreactive NK cells and lymphokine-activated killer cells and/or antigen-specific $CD8^+$ T cells. This protective effect could be reversed by masking HLA-G antigens using a HLA-G1-specific antibody. In ovarian carcinoma, the resistance to lysis by peptide- and allo-specific $CD8^+$ T cells was not due to HLA-G, but reflects its ability to induce HLA-E expression in tumor cells. Furthermore, $HLA\text{-}G^+$ melanoma cells have been found to express membrane-bound, and secrete, HLA-G. Additional mechanisms may also trigger NK cell-mediated tumor cell

lysis. These include the stress-inducible molecules MIC-A, which have been reported to be selectively switched on in various tumors of epithelial and non-epithelial origin. There exists a balance between the activating signal delivered by MIC-A and the inhibitory signal generated by HLA-G1 on NK cell-mediated lysis of melanoma cells [131]. These data suggest that, in vivo, the over-expression of inhibitory ligands by tumor cells, such as HLA-G, may bypass activating signals mediated by MIC-A thereby favoring tumor progression.

Conclusions

With the availability of respective tools, a high frequency of HLA-G surface expression and increased sHLA-G serum levels have been detected in hematological and solid tumors of distinct origin, which often correlate with a poor clinical outcome of tumor patients. This might be due to its immune suppressive effects demonstrated in in vitro studies, suggesting that HLA-G is an important immune escape mechanism of tumors. Although many studies have been hampered by the small number of tumor lesions and plasma/serum samples analyzed, it might serve as a diagnostic and prognostic biomarker to distinguish between benign and malignant tumors and to identify tumors with worse prognosis. However, the application of HLA-G asa biomarker has to be validated by analyzing a large series of tumor samples and patients' serum.

Due to its broad expression, HLA-G might also serve as a therapeutic target for cancer patients, which will be addressed as a long-term goal.

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