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Soluble HLA-G Molecules are Increased in Lymphoproliferative Disorders

Yasmine Sebti, Gaëlle Le Friec, Céline Pangault, Frédéric Gros, Bernard Drénou, Valérie Guilloux, Marc Bernard, Thierry Lamy, Renée Fauchet, and Laurence Amiot

ABSTRACT: The immunomodulatory properties of soluble human leukocyte antigen G (sHLA-G) explain its potential interest in malignancies. HLA-G frequently transcribed in lymphoproliferative disorders is rarely expressed at cell surface. In this article, we will demonstrate that the plasmatic level of soluble HLA-G was significantly increased in 70% of B chronic lymphocytic leukemia, 53% of non-Hodgkin B lymphoma (B-NHL), and 45% of T-NHL. To explain this variable secretion, the HLA-G secreting cell was searched and was identified as tumoral T4 lymphocytes only in one patient with Sezary syndrome. To approach the mechanisms involved in sHLA-G secretion, the potential role of cytokines has been studied *in vitro* on T lymphomas. A significant increase of sHLA-G level is observed after activation by cytokines associated with a small increase in the quantity of transcripts using real-time polymerase chain reaction, suggesting an involvement of both transcriptional and post-transcriptional mechanisms. Western Blot analysis reveals no evident variation of the protein expression whatever the conditions, suggesting a continuous secretion and a low intracellular storage. The frequency of the sHLA-G secretion associated to its inhibiting role on T cells and natural killer cells during tumoral lymphoid malignancies suggests a potential role of these molecules as escape mechanism from antitumoral response. *Human Immunology 64, 1093–1101 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Inc.

KEYWORDS: soluble HLA-G; ELISA; lymphoproliferative disorders; T lymphoma; Sézary syndrome; cytokines

ABBREVIATIONS

CLL	chronic lymphocytic leukemia
NHL	non-Hodgkin lymphoma
HLA	human leukocyte antigen
sHLA-G	soluble HLA-G

INTRODUCTION

Human leukocyte antigen G (HLA-G) is a nonclassical major histocompatibility complex (MHC) class I gene characterized by a restrictive distribution [1–3], a limited polymorphism [4], and an alternative splicing leading to at least seven isoforms including four membrane-bound forms (HLA-G1, -G2, -G3, -G4) [5, 6] and three soluble forms (HLA-G5, -G6, -G7) [7, 8]. In contrast to classical MHC class I molecules, HLA-G appears to function as a mediator of immune tolerance during pregnancy and also during immune reactions. The functional properties of membrane and soluble isoforms that explained the immune regulatory role of HLA-G consist of inhibiting the function of natural killer (NK) cells and T8 lymphocytes by the following two mechanisms: (i) the membrane-bound isoforms are able to inhibit cytotoxicity of NK cells and T lymphocytes through direct [9–13] or indirect [14, 15] interaction with several inhibitory receptors; and (ii) the soluble isoforms like soluble MHC class I proteins are capable of inducing apoptosis of these cytotoxic cells through CD8 ligation [16–18]. Thus, the complete membrane-bound isoform HLA-G1 and its soluble counterpart HLA-G5 are able to

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reduce alloproliferation of T lymphocytes [19], to interfere with effective priming of antigen-specific cytotoxic T cells, and to reduce antigen-specific alloreactive lysis in the models of myoblasts and gliomas [20, 21]. These immunomodulating properties explain the importance of HLA-G expression or secretion during pregnancy, and probably during transplantation and tumoral processes.

In contrast to soluble MHC class I molecules, the special feature of soluble HLA-G molecules is to be generated by an alternative splicing with intron persistence. Three soluble isoforms have been identified to date: HLA-G5, -G6, and -G7 [7, 8]. The soluble forms HLA-G5 and -G6 are characterized by retaining part of intron 4 that contains a stop codon preventing the translation of the transmembrane and cytoplasmic domains. Thus they present respectively three ($\alpha 1$,$\alpha 2$, $\alpha 3$) or two ($\alpha 1$,$\alpha 3$) external domains. Their structure allows their potential association with the light chain, the beta-2 microglobulin through the $\alpha 3$ domain. The HLA-G7 isoform has been more recently identified and was characterized by a stop codon in nonspliced intron 2, producing a smaller isoform displaying only $\alpha 1$ domain. Soluble HLA-G (sHLA-G) molecules have been detected in amniotic fluid, in cord blood, and in peripheral blood. During pregnancy, sHLA-G was expressed by extravillous cytotrophoblast cells invading the decidua and was found to be secreted by human villous trophoblast [22]. In plasma, a basal level has been defined in healthy patients with no significant difference between males and females [23], however, the type of cell secreting HLA-G was not yet identified. In pathologic situations HLA-G soluble has been found in the graft and serum of heart transplanted patients [24, 25]. HLA-G was previously demonstrated to be expressed in some tumors, such as melanomas [26], renal [27], breast [28], lung carcinomas [29], gliomas [20], or cutaneous lymphomas [30]. However, the respective role of membrane and soluble isoforms was not clearly defined in these pathologic situations. The plasma level of sHLA-G has not been studied during malignancies, except in melanomas where it was reported to be elevated [31]. If the plasma level of these soluble forms is increased during malignancies, this appears to be a relevant question because they display immunotolerant functions similar to membrane-bound forms, subsequently favoring tumoral escape from immune system. In lymphoproliferative disorders, defined as monoclonal proliferation of B or T lymphoid cells, we have previously demonstrated the rarity of HLA-G cell surface expression [32] contrasting with the frequency of the transcription of the different mRNA isoforms [33, 34]. We consequently investigated the plasma level of HLA-Gs during lymphoproliferative disorders including B chronic lymphocytic leukemias (CLL), and B or T non-Hodgkin lymphomas (NHL). In this study, we revealed that the plasma level of sHLA-G was significantly increased compared with healthy patients. We also demonstrated the role of cytokines in induction of HLA-G secretion by T lymphomatous cells using *in vitro* experiments.

MATERIAL AND METHODS

Patients

One hundred and three patients suffering from lymphoproliferative disorders from the Department of Hematology in the Rennes's hospital (Dr. Lamy) were studied. These disorders were classified according to the OMS classification using histologic, immunophenotypical, including Matutes scoring, molecular and cytogenetical criteria. They consisted of 17 CLL patients, 75 B-NHL patients, and 11 T-NHL patients. Plasma was obtained after centrifugation at 1000*g*, 4 °C of EDTA-anticoagulated blood provided from the patients. Plasma of healthy patients ($n = 30$), provided by EFS Bretagne, were used as controls and were cryopreserved at −80 °C. Mononuclear cells from lymphoproliferative disorders were isolated by Ficoll density gradient centrifugation and cryopreserved at −180 °C.

Cytokines

Interleukin-2 (IL-2; 10^4 U/μg), interferon-γ (IFN-γ; 1×10^7 U/mg), and IL-10 (> 5×10^5 U/mg) were supplied by Peprotech-Tebu (Le Perray en Yvelines, France); human granulocyte macrophage–colony-stimulating factor (GM-CSF; sp act 1.2×10^8 U/mg) was provided by Shering Plough (Lyon, France).

Cell Cultures

Mononuclear cells were purchased from nine cryopreserved patients of T-NHL, including six Sézary syndrome patients, 1 patient with T/NK NHL, 1 angioimmunoblastic NHL patient, 1 patient with T peripheral lymphoma, and were characterized by more than 80% tumoral cells. Cells were cultured during 48 hours in RPMI medium supplemented with 2-mM glutamine, 1% pyruvate, antibiotics, and 10% fetal calf serum in the presence and absence of different combinations of cytokines. The different conditions were as follows: IL-2 (100 U/ml) and IFN-γ (500 U/ml); IFN-γ (100 U/ml), IL-2 (220 U/ml), and GM-CSF (100 U/ml); and IFN-γ (500 U/ml) with GM-CSF (100 U/ml) or IL-10 (50 ng/ml).

RT-PCR Analysis

Total RNA was isolated from 1 to 3×10^6 cells using TRIzol reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's recommendations. cDNA synthesis was performed on 5-μg RNA by random hexamers priming using Superscript II reverse transcriptase

(Invitrogen) for 50 minutes at 42 °C. After denaturation of enzyme at 70 °C for 15 minutes, RNA template was digested by adding RNAse H for 20 minutes at 37 °C. Hot start PCR was carried out in a 50-μl volume containing 10-μl RT reaction, 50-pmol each primers, 10 μl of 10× PCR buffer, and 2.5-U Taq polymerase (Amersham Pharmacia Biosciences, Uppsala, Sweden). The following primer sets were used: G257 (exon 2; 5′-GGAA GAGGAGACACGGAACA)/G1004 (exon 5 and exon 6 junction; 5′-CCTTTTCAATCTGAGCTCTCTTT). PCR cycle conditions were 1 minute at 94 °C, 1.5 minutes at 61 °C, and 1.5 minutes at 72 °C for 35 cycles, with a final extension at 72 °C for 10 minutes. Beta-actin cDNA was amplified in the same conditions to ensure RNA quality of each sample. PCR products were separated in an ethidium bromide stained 1.7% agarose gel. Alkaline DNA transfer was performed under vacuum on Hybond-N^+ membrane. Southern blot was prehybridized 1 hour at 68 °C, and then hybridized 3 hours at 57 °C in buffer containing 5′ digoxigenin-labeled GR probe (exon 2 specific; 5′-GGTCTGCAGGTTCATTCTGTC). Revelation was performed using anti-digoxigenin system.

Real-Time Quantitative PCR

Duplex PCR was carried out for 40 cycles in presence of Taqman universal PCR master mix on an ABI prism 7000 (Applied Biosystem, Foster City, CA, USA) using HLA-G specific primers, HLA-G specific probe (FAM reporter and TAMRA quencher), GAPDH as endogeneous control (VIC reporter and TAMRA quencher; Applied Biosystems) as previously described [35]. Quantifications relative to JEG3, an HLA-G expressing choriocarcinoma cell line, were carried out in duplicate, using the following calculations: $\Delta_{C\ t} = C_{T\ HLA\text{-}G} - C_{T\ GAPDH}$, and $\Delta\Delta Ct = \Delta_{CT\ sample} - \Delta_{CTJEG3}$. The quantity of HLA-G level is defined as $2^{-\Delta\Delta CT}$.

Specific Soluble HLA-G Enzyme-Linked Immunosorbent Assay

Soluble HLA-G concentrations were measured using a specific sandwich enzyme-linked immunoabsorbent assay (ELISA). Microtiters plates (Corning Costar, Issy-les-Moulineaux, France) were coated with MEM-G/9 (Exbio, Prague, Czech Republic; 10μg/ml) in 0.01-M PBS, pH 7.4. After three washes in PBS containing 0.05% Tween 20, plates were saturated with 250-μl PBS containing 2% BSA for 30 minutes at room temperature. Cell culture supernatants or sera (100 μl) were added to each well and were tested in triplicate. After incubation for 1 hour at room temperature, plates were washed three times in PBS with 0.05%Tween 20. Anti β_2-microglobulin HRP (Dako, Trappes, France; 100μl) was added to each well and plates were incubated for 1 hour at room temperature. Plates were washed three times and then incubated with the substrate (ortho-phenylenediamine dihydrochloride; Dako) for 30 minutes. After the addition of H_2SO_4 (1N), optical densities were measured at 490 nm. Standard curves were performed using serial dilutions of calibrated supernatant of LCL-G5 kindly provided from V. Rebmann (Essen, Germany). Thus, the concentrations of sHLA-G were determined from the value of optical density according to the standard curves.

Immunocytochemistry

Cells (1.5×10^5) were cytocentrifuged on glass slides. Samples were stored wrapped in aluminium foil at −20 °C until use. Staining procedures were processed at room temperature using LSAB-2 Kit phosphatase alkaline (Dako). After fixation in cold acetone and rehydratation in tris-buffered saline (TBS; pH 7.4), potential Fc receptor binding was blocked by incubation in 3%BSA–40% human AB-group serum in TBS for 20 minutes. Samples were then incubated with the 87 G (5 μg/ml), MEM-G/9 (5 μg/ml) monoclonal primary antibodies or isotype-matched controls for 10 minutes. Bound 87 G or MEM-G/9 was detected by sequential incubations with biotinylated antimouse antibodies, alkaline phosphatase-conjugated streptavidin, and substrate chromogen (red fushine). To identify the type of HLA-G positive cells, the same preparations were saturated once again by 3% BSA–40% human AB-group serum in PBS and then incubated for 10 minutes with the anti-CD4 to detect T cells. Binding was detected by sequential incubations with biotinylated antimouse antibodies, alkaline phosphatase-conjugated streptavidin, and substrate chromogen (Vector blue; Vector Laboratories, Burlingame, CA, USA). The immunostained samples were mounted in aqueous mounting medium (Aquatex; Merk, Kriftel, Germany) and analyzed by light microscopy.

Western Blotting

Proteins from cell lines (JEG3, LCL721.221-G5) and from T-NHL cultured under three different conditions (control, IFN-γ and IL-2 and GM-CSF, and IL-10) were extracted using a lysis buffer containing 1% Nonidet P-40 and 0.5% sodium deoxycholate, and were then sonicated for 30 seconds. Solubilized proteins were loaded in a 12% mini-SDS-PAGE and transferred onto a PVDF membrane using an electrophoretic transfer apparatus. Membranes were saturated with 5% non-fat dry milk in PBS and incubated with the 16G1 mAb (4 μg/ml). A peroxydase-conjugated anti IgG mouse was thereafter used as secondary antibody. After washing, blots were developed by chemiluminescence using a 100-mM Tris-HCl solution (pH 8.5) containing 0.9% (w/w) H_2O_2, 225-μM coumaric acid, and 1.25-mM luminol.

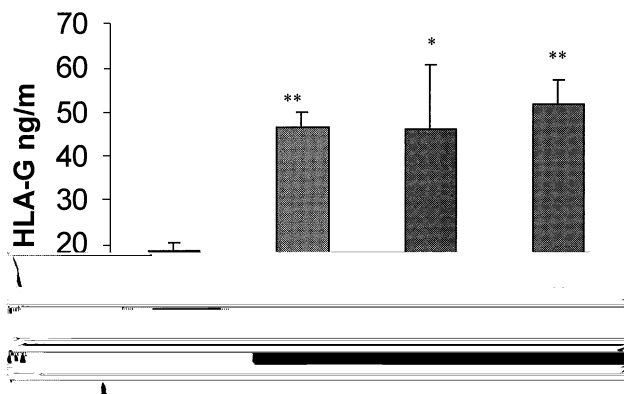

FIGURE 1 Level of sHLA-G in lymphoproliferative disorders compared with healthy patients. The sHLA-G level is illustrated as histograms with standard errors in CLL (n = 17), B-NHL (n = 75), and T-NHL (n = 11) compared with healthy patients (n = 30). The sHLA-G level in CLL, B-NHL, and T-NHL are significantly increased: $*p < 0.005$ and $**p < 0.001$. Abbreviations: CLL = chronic lymphocytic leukemia; NHL = non-Hodgkin lymphoma; sHLA-G = soluble human leukocyte antigen G.

Flow Cytometry

Cells (5×10^5) were incubated with the anti-HLA-G 87G (kindly provided by D. E Geraghty; 10μg/ml) or MEM-G/9 (10μg/ml) recognizing HLA-G1 and -G5 for 30 minutes at 4 °C. Murine isotype matched control respectively IgG2a and IgG1 were used as negative controls. After washing, cells were incubated for 30 minutes at 4 °C with a goat antimouse IgG (Fab'2) fraction conjugated with phycoerythrin. Fluorescence was detected by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

RESULTS

Increased Soluble HLA-G in Plasma of Patients Suffering From Lymphoproliferative Disorders

The plasma level of sHLA-G was 18.57 ± 9.29 ng/ml in healthy patients (n = 30). The threshold to define an increase level of sHLA-G was the mean of the basal level ± 2 standard deviations of the mean (> 37.17 ng/ml). The mean of sHLA-G was generally significantly increased (Figure 1) in B-CLL (n = 17, 51.47 ± 23.56 ng/ml, $p < 0.001$), in B-NHL (n = 75, 46.42 ± 29.95 ng/ml, $p < 0.001$), and in T-NHL (n = 11, 45.78 ± 48.66 ng/ml, $p < 0.005$). However, the plasma level of sHLA-G is not elevated in all patients suffering from lymphoproliferative disorders because an increased level is observed only in 12 of 17 CLL patients, in 40 of 75 B-NHL patients, and in 5 of 11 T-NHL patients, the others display a normal level of soluble HLA-G molecules. In the group of patients with elevated sHLA-G, the mean of sHLA-G is 63.03 ± 16.9 ng/ml (range 39.32–93.12) in CLL, 67.41 ± 26.09 ng/ml (range, 38.15–141.5) in B-NHL, and 78.91 ± 58.05 ng/ml (range, 37.3–181.4) in T-NHL.

Secreting Cell Was Not Evidenced in Lymphomatous Cells Except in One Patient

Flow cytometry with 87G and MEM-G/9 was performed in the 17 CLL patients, 75 B-NHL patients, and 11 T-NHL patients. Immunocytochemistry using 87G or MEM-G/9 (Exbio), were performed in certain cases of this set, *i.e.*, 2 CLL, 11 B-NHL, and 5 T-NHL. No HLA-G expressing cell was evidenced in all tested patients except in one patient with Sezary syndrome, characterized by a very high plasma level (500 ng/ml) of sHLA-G (Figure 2). In this patient HLA-G expression was detected at cell surface using flow cytometry as well as in the cytoplasm of tumoral cells using immunocytochemistry. Double labeling with anti-CD4 monoclonal antibody (mAb) confirms the positivity of tumoral T4 cells.

sHLA-G Secretion Is Induced *In Vitro* After Stimulation of T Lymphomatous Cells With Cytokines

Nine cases of mononuclear cells provided from T-NHL were cultured for 48 hours in presence and absence of different combinations of cytokines. sHLA-G secretion was significantly increased in supernatants of cultures after 48 hours of cytokinic activation. The results are illustrated in Figure 3. The combination allowing to obtain the stronger secretion is variable according to the patients, even if IL-10 or the association of IL-2, IFN-γ, and GM-CSF appears to be more effective, respectively, in 2 and 3 patients.

The effect of these combinations of cytokines was also investigated at mRNA and protein level. Using RT-PCR and Southern blotting, with primers and probe previously validated [36], no apparent variation in the m RNA transcription was observed in presence and in absence of cytokines (n = 3; Figure 4A) with at least five transcriptional isoforms (HLA-G1, HLA-G5, HLA-G6, HLA-G2/G4, and HLA-G3). To analyze the quantitative variation of HLA-G transcripts, quantitative real-time PCR was performed using primers and probe previously described [35]. The results are expressed in percentage of the transcripts found in the positive cell line JEG3, used as calibrator with an assigned value of 100%. Despite a low HLA-G transcription in T-NHL, compared with the JEG3 cell line, we demonstrated a significant increase of HLA-G transcription in the presence of cytokines compared with the absence (fivefold upregulation). Moreover, the protein expression (n = 2) was characterized using Western blot with 16G1 as blotting mAb recognizing

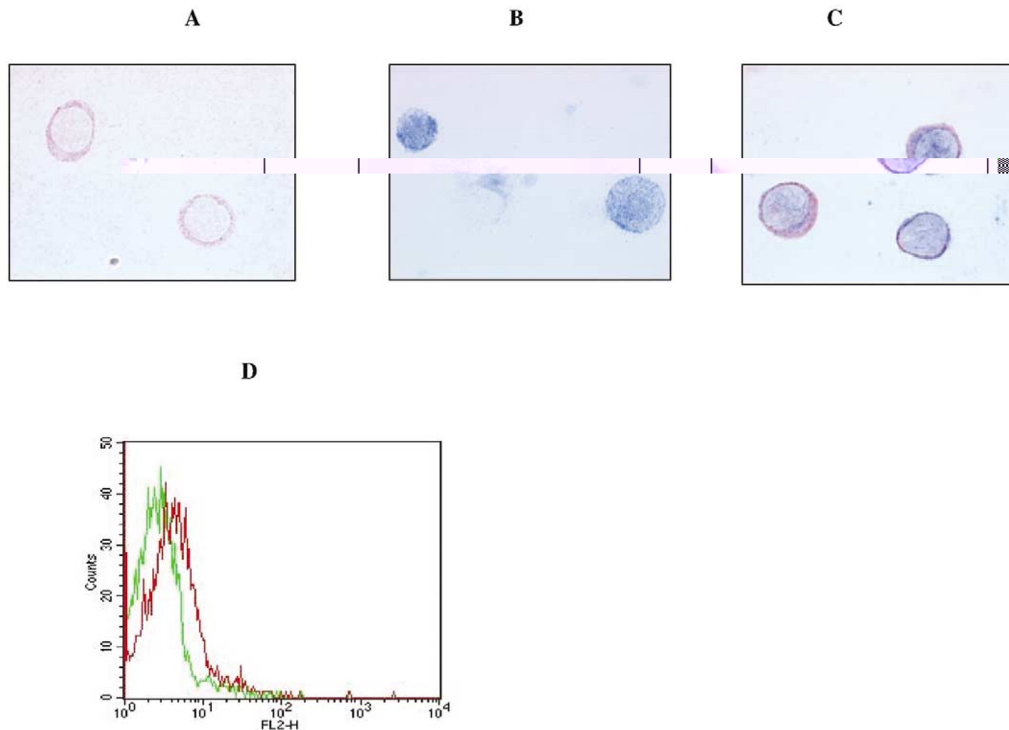

FIGURE 2 Soluble human leukocyte antigen G (sHLA-G) is secreted by tumoral T lymphocytes in a case of Sezary syndrome (magnification ×630). Immunocytochemistry and flow cytometry analyses are represented, respectively, in panels A, B, C, and D: (A) using single labeling with MEM-G/9, HLA-G positive cells are stained in red; (B) using single staining with anti-CD4 mAb, CD4 T cells are stained in blue; (C) using double labeling with MEM-G/9 and CD4 mAbs, CD4 cells expressing-HLA-G were stained both in blue and red; and (D) this flow cytometric graph, using MEM-G/9, illustrates a weak shift between MEM-G/9 in red and isotypic control in green.

soluble forms HLA-G5 and -G6 in three conditions: control; IL-2, IFN-γ, and GM-CSF; and IL-10. The 37-kDa band corresponding to HLA-G5 is obtained only for LCL.721.221-G5, whereas a single band of 45 kDa is observed in the two tested patients, similar to one of the bands obtained from JEG3 and LCL.721.221-G5. The intensity of the band was weak whatever the condition and seems to increase slightly in presence of cytokines (Figure 4B).

DISCUSSION

HLA-G expression is variable in lymphoproliferative disorders according to the level of detection (*i.e.*, transcription), protein expression, and secretion of soluble forms. In this work, we have investigated the plasmatic level of soluble isoforms in this type of malignancy and examined the influence of cytokines on this secretion in T-NHL. HLA-G mRNA are present in the majority of the pa-

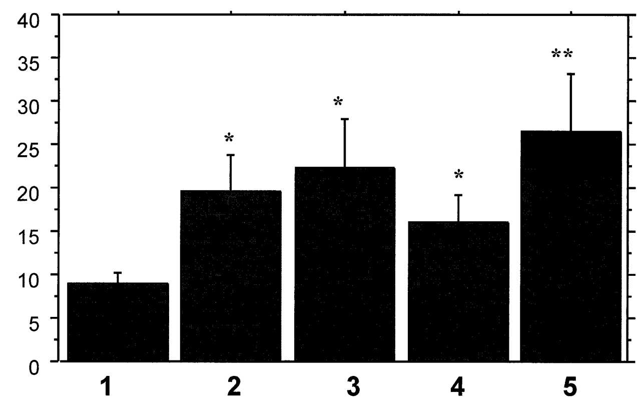

FIGURE 3 Increase of sHLA-G level after cytokines stimulation of T-NHL. Mononuclear cells providing from T-NHL (n = 9) are cultured for 48 hours in five different conditions: (bar 1) absence of cytokines; (bar 2) IFN-γ and IL-2; (bar 3) IFN-γ, IL-2, and GM-CSF; (bar 4) IFN-γ and GM-CSF; and (bar 5) IL-10. The mean of sHLA-G level measured by specific ELISA is represented as histograms. Significant increase of sHLA-G in medium culture is observed in presence of cytokines (bars 2, 3, 4, and 5) compared with controls (bar 1): *$p < 0.05$ and **$p < 0.01$. Abbreviations: ELISA = enzyme-linked immunosorbent assay; GM-CSF = granulocyte macrophage–colony-stimulating factor; HLA = human leukocyte antigen; IFN = interferon; IL = interleukin; NHL = non-Hodgkin lymphoma; sHLA-G = soluble HLA-G.

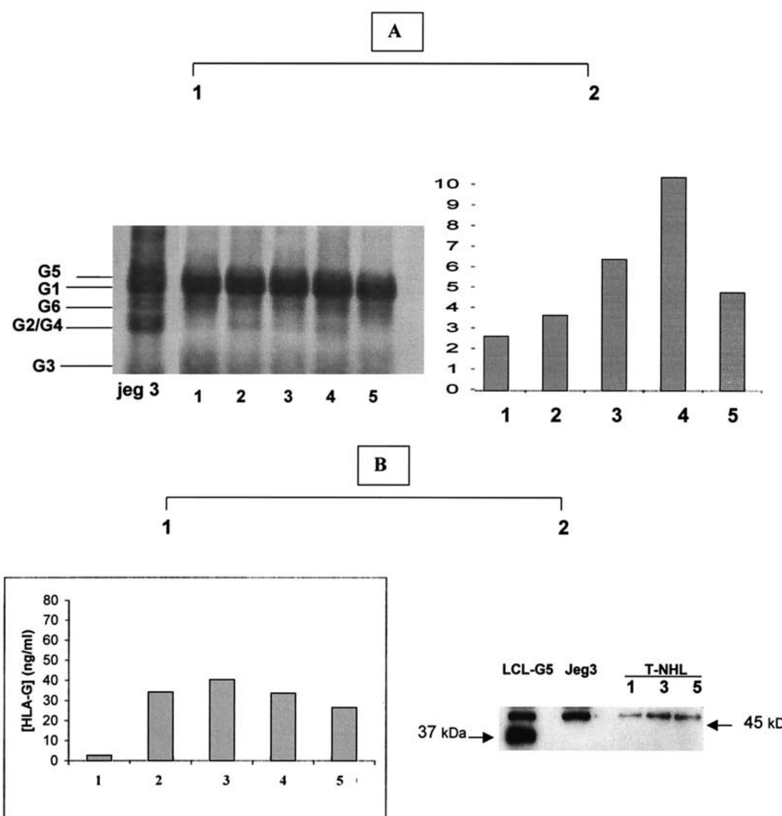

FIGURE 4 Transcriptional, protein, and secretion pattern analyses of one representative T-NHL patient stimulated by cytokines. The conditions were as follows: 1 = absence of cytokines; 2 = IFN-γ and IL-2; 3 = IFN-γ, IL-2, and GM-CSF; 4 = IFN-γ and GM-CSF; 5 = IL-10. (A) Corresponds to transcriptional analysis comprising in (1) RT-PCR/Southern blot and in (2) real-time PCR; (B) corresponds to protein analysis including (1) sHLA-G analysis by ELISA in the different conditions, and (2) Western Blot analysis with 16G1 in conditions 1, 3, and 5. In A1, Jeg 3 is used as positive controls, the different isoforms (G5, G1, G6, G2/4, G3) are indicated by arrows. Several transcripts are observed in all conditions with no detectable change. In A2, an increase of the quantity of transcripts is observed in conditions 2, 3, 4, and 5 when compared with condition 1. In B1, an increase in the sHLA-G level is detected in all conditions (2, 3, 4, and 5). In B2, Jeg3 and LCL721-221-G5 are used as positive controls 1, 3, and 5 and are corresponding to the T-NHL study according to the 1, 3, and 5 conditions. A band of 37 kDa is only observed in LCL-G5, whereas a band of 45 kDa is observed with strong intensity in LCL-G5, Jeg3; and with a low intensity in the T-NHL in the various conditions. Abbreviations: GM-CSF = granulocyte macrophage–colony-stimulating factor; HLA = human leukocyte antigen; IFN = interferon; IL = interleukin; NHL = non-Hodgkin lymphoma; sHLA-G = soluble HLA-G.

tients through numerous isoforms. These alternatively spliced mRNA forms are variably distributed with no specific pattern according to the pathology. In contrast, the cell surface expression of the protein is rarely observed, suggesting post-transcriptional mechanisms of regulation and/or expression of another type of isoform, such as soluble isoforms. The soluble molecules have been evidenced in plasma of healthy patients and in amniotic fluid using specific sandwich ELISA [22, 23], and have been only reported increased in patients with melanomas [31]. Our data reveals a significant increase of the plasmatic level of soluble HLA-G in 70% of CLL, in 53% of B-NHL, and in 45% of T-NHL patients. The level of increase does not appear to be statistically different ($p < 0.8$) according to the type of lymphoproliferative disorder (CLL, B-NHL, T-NHL). However, this ELISA method did not allow discrimination between alternative spliced HLA-G5 and soluble shedded HLA-G1.

Despite its frequency in these pathologies, HLA-G secretion appears not to be a constant feature, suggesting the implication of other mechanisms of induction. To approach these mechanisms, we first searched to identify the cell population responsible for HLA-G secretion using flow cytometry and immunocytochemistry. No cell expressing HLA-G was found except in a Sezary syndrome patient, characterized by an exceptionally strong level of soluble HLA-G. These cells, evidenced by double immunocytochemistry, were tumoral T4 lymphocytes. On the other hand, no B tumoral cells expressing HLA-G were found using this methodology. This finding is consistent with a recent publication demonstrating

a possible HLA-G secretion by CD4 T lymphocytes during allogeneic reaction [19].

To further investigate the potential factors acting on these T cells, we assessed the role of cytokines on HLA-G secretion in these T lymphoid malignancies. Indeed the roles of cytokines have been demonstrated in several models. First, IFN-γ was reported to induce HLA-G protein expression in human gliomas [20], in myoblasts [37], and in ovarian carcinoma cells [38]. The potential synergy between IFN-γ and other cytokines, such as IL-2, GM-CSF has been found in monohistiocytic U937 cell line [32]. More recently, the role of IL-10 in inducing HLA-G protein expression has been demonstrated in monocytes and purified trophoblast cells [39], and the association between IL-10 and HLA-G expression was demonstrated in lung carcinomas [29]. The mechanism of cytokines regulation appears variable depending on the type of cytokines and on the cell type. Analysis of sHLA-G level in supernatants of mononuclear cells providing from T-NHL reveals an increase in all the tested patients ($n = 9$) when stimulating with cytokines. In contrast, no sHLA-G induction was found on normal mononuclear cells comprising 70% of T lymphocytes stimulated under similar conditions ($n = 4$; data not shown). The differential effect on normal and tumoral T lymphocytes could be explained by a different susceptibility induced by tumoral process. Indeed, it was demonstrated that an association between cytokines profile and Sezary syndrome [40], defined as an advanced form of cutaneous T-cell lymphoma, associated with involvement of peripheral blood by malignant T cells. This disease is characterized by a decrease of Th1 cytokines switching to Th2 response. Because IL-10 is able to suppress the local immune response, its production has been reported as an immune escape mechanism, in particular in NK/T lymphomas [41]. On the other hand, the association between HLA-G and IL-10 expression was previously demonstrated in B and T cutaneous lymphomas [30]. Moreover, it remains to be determined whether the action of cytokines is solely targeted directly on T tumoral cells or was only partial by effect on residual cells. This last hypothesis is suggested by the efficiency of association of cytokines, including GM-CSF, which is rather a cytokine involved in myelo-monocytic differentiation. The level of cytokinic regulation was approached by transcriptional analysis using two approachs. The first one consisted of standard RT-PCR/Southern blot that informed qualitatively on the presence of transcription and on the type of transcriptional isoforms. The second method was real-time PCR detecting all HLA-G transcripts, which allowed detection of the global quantity of transcripts like the variation in their amount compared with a calibrator.

Standard analysis revealed a transcription of several alternative m RNA forms with no apparent change according to the condition because real-time PCR demonstrated an increase after stimulation in the quantity of total transcripts by a fivefold factor when compared to the condition with no stimulation. However, HLA-G transcript levels even after cytokinic stimulation remained weak compared to those observed in JEG3. These findings seem to indicate that no correlation exits between the quantity of transcripts and the protein detection, confirming the dissociation between HLA-G transcription and expression. Thus the difference between the low increase of transcript amount and the strong augmentation of the level of sHLA-G may be in favor of post-transcriptional mechanisms in addition to transcriptional regulation. This hypothesis is reinforced by the results of Western blot analysis using 16G1, which illustrated no clear difference in the quantity of soluble protein in the cells providing from T-NHL whatever the conditions. Surprisingly, the molecular weight of the bands obtained from T-NHL at basal condition and after stimulation by IL-2, GM-CSF, and IFN-γ or IL-10 was approximately 45 kDa, similar to JEG3, instead of the expected 37-kDa band. This 37-kDa band corresponding to the weight of soluble form was only observed in LCL721.221-G5. The 45-kDa band was also found in the transfected cell line, suggesting that soluble HLA-G molecules are subjected to glycosylation. The involvements of potential glycosylations prior secretion have been previously reported in purified villous trophoblast cells [22]. The absence of a detectable increase in quantity of a detected protein may account for a high rate of secretion with a lack of storage within the cell, as described in certain models. This secretory pattern may explain the difficulty in detecting the cell-secreting HLA-G beside a strong HLA-G secretion.

These soluble HLA-G forms may constitute a new way of immune escape. Indeed, the negative immune regulatory effects of soluble HLA-G appear to be similar to membrane forms because they are able to inhibit the functions of T lymphocytes and NK cells playing a key role in antitumoral response. These mechanisms previously described are as follows: (i) induction of apoptosis of T lymphocytes and NK cells in contrast to membrane-bound forms [16–18]; (ii) reduction of the T allogeneic lymphocytes [19]; and (iii) inhibition of the priming and the antigen specific lysis by cytotoxic T lymphocytes [20, 21]. In contrast to membrane-bound forms, the increase of soluble forms is frequently observed during the tumoral lymphoid malignancies and thus presents clinical interest. The analysis of correlation between clinical and biologic data during prolonged period and plasma sHLA-G level would allow appreciation of its significance. Understanding the mechanisms underlying the appearance of these soluble forms should be impor-

tant in modulating negative or positive sHLA-G, respectively, in malignancies or transplantations.

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