HLA-G levels in serum and plasma

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Key words
ELISA; HLA-G; serum; plasma

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Received 24 May 2005; revised 18 August 2005; re-revised 25 September 2005 & 02 November 2005; accepted 09 November 2005

doi: 10.1111/j.1399-0039.2005.00540.x

Abstract

HLA-G belongs to the non-classical HLA class-I family of genes presently designated as class-Ib genes. There are four membrane-bound (HLA-G1 to -G4) and three soluble forms (HLA-G5 to -G7) generated by alternative splicing of the primary transcript. HLA-G in the soluble form is found in the plasma, amniotic fluid, and cord blood of healthy individuals. Quantitative determination suggested that HLA-G levels are genetically controlled. While quantifying soluble HLA-G by ELISA, we observed that when plasma and serum levels were measured for the same individual, HLA-G plasma values were almost invariably higher than those from serum. Our results suggest that HLA-G is trapped and/or consumed during clot formation. The amount trapped within the clot is variable and inconsistent. To obtain values which reflect the true biological levels, it is therefore recommended that HLA-G should be determined in the plasma. If serum levels are determined, they should be compared with matched control sera. It should always be borne in mind that conclusions concerning sera levels might be erroneous, because the true plasma level of the protein can be significantly higher.

Introduction

HLA-G belongs to the non-classical HLA class-I family of genes presently designated as class-Ib genes [see review (1)]. It was first described as an HLA class-I gene that encodes a short cytoplasmic tail (2). Subsequently, it was reported that it is subjected to alternative splicing, yielding proteins with structures similar to HLA class-I and class-II antigens (3). It was found to be expressed mainly in placental tissue and in the thymus (4–8). A unique feature of HLA-G is the presence of a stop codon in exon 6, which shortens its cytoplasmic protein tail (3–8). There are four membrane-bound (HLA-G1 to -G4) and three soluble forms (HLA-G5 to -G7) generated by alternative splicing of the primary transcript (9, 10). Limited polymorphism of HLA-G and expression restricted to the cytotrophoblast (11, 12) prompted the hypothesis that it provides the barrier that shields the fetus from the innate and/or acquired immunity of the mother. However, subsequent studies showed that this cannot be the exclusive mechanism of materno-fetal tolerance (13). Other studies showed that basal levels of HLA-G are transcribed in almost all tissues examined (14, 15). HLA-G in the soluble form is found in the plasma, amniotic fluid, and cord blood of healthy individuals (16). Quantitative determination of its levels suggested that the level is genetically controlled (17, 18).

Many studies were aimed at the determination of HLA-G in the plasma and/or serum of patients suffering from various diseases. Soluble HLA-G levels were determined in habitual abortions, in autoimmunity, in solid organ transplantation, and in various malignancies (19–30). In some studies, levels were found to be higher when compared with the control cohort, while in other studies levels were low when compared with controls. Because the enigma of HLA-G has not, as yet been solved, most studies were aimed at finding an explanation for its function in health and disease [16, 29, 31–34].

While quantifying soluble HLA-G by ELISA, we observed that when plasma levels were measured, HLA-G values were almost invariably higher than in random serum samples. Hence, the following studies were carried out to explore the reason for such variability. Our results suggest that HLA-G is trapped during clot formation. The amount trapped within the clot is variable and inconsistent. To obtain values which reflect the true biological levels, it is therefore recommended that HLA-G should

be determined in the plasma. However, if only serum samples are available for a study, they should be compared with matched serum controls. Conclusions concerning HLA-G levels might be erroneous, because the true plasma level of the protein can be significantly higher.

Materials and methods

Blood samples

Blood samples were drawn from healthy volunteers after signing an informed consent form. Venous blood aliquotes from the same individual were placed into tubes containing dry EDTA, and glass tubes without anticoagulants. The latter were left at room temperature for 2–3 h for clot formation, and the serum was subsequently separated by centrifugation. EDTA-plasma and sera were kept frozen at −30 °C until used.

Recalcification of plasma

Recalcification of EDTA plasma was carried out by the addition of $CaCl_2$ (1 M, 20 μl) to 1 ml of plasma and incubated at 37 °C. If clot did not form, two drops of thrombine (Thrombine Topical-bovine, 200 μ/ml Armour Pharmaceutical Company, Kankakee, IL, USA) were added, and incubation continued until a clot was visible. The serum was subsequently separated by centrifugation.

Tissue Plasminogen Activator treatment

Recombinant tissue plasminogen activator (TPA) (25 μl of balanced salt solution containing 0.25 mg/ml TPA) was added to glass tubes containing 1 ml of clotted whole blood and incubated in a water bath at 37 °C for 30 min. The tubes were shaken vigorously every 10 min. Following incubation, the released serum was separated by centrifugation and kept frozen until used.

ELISA procedure

Microtiter trays (immunoplate, maxisorp, cat. no. 442404, Nunc, Roskilde, Denmark) were coated with the capturing antibody MEM-G/9 MoAb (10 μg/ml) diluted in phosphate buffered saline (PBS), 100 μl/well. MEM-G/9 is a monoclonal antibody which binds to soluble HLA-G5 and to the cell bound and shedded HLA-G1 isoforms (34). Coating was carried out at 37 °C for 2 h. The trays were washed four times with PBS containing 0.2% Tween-20. Subsequently, blocking was performed for 2 h at 37 °C with 2% bovine serum albumin in PBS. After four washings with cold 0.2% Tween-20 in PBS, the sera to be tested were added, in duplicate, 100 μl/well diluted 1:2 in PBS and incubated overnight at 4 °C. Dilution of 1:2 was used, because such dilutions were used in most other reports. In addition, dilution reduces the viscosity of the serum/plasma and enhances specific binding. Following four washings with 0.2% Tween-20 in PBS, goat anti β-2-microglobulin/Horse-radish peroxidase (HRP) (Dako cat. no. P0174, Dako Denmark) diluted 1:500, 100 μl/well were added and incubated at 37 °C for 60 min. This was followed by four washings with PBS/Tween-20. The enhancer, Envision System (Dako Envision system HRP, cat. no. K4002), diluted 1:15 in PBS, 100 μl/well was added. Incubation was performed for 20 min at room temperature and was followed subsequently by five washings with 0.2% Tween-20 in PBS, and then 100 μl of the substrate Tetra-methyl-benzidine (TMB) (Dako cat. no. S1599) was added. The trays were incubated in the dark at room temperature for 15–20 min. The reaction was terminated by the addition of 100 μl HCl 1 N. The OD (450 nm) of the reactions was read by an ELISA reader and quantified by comparing the optical density to a reference serum whose concentration was determined by a calibration curve (Figure 1) (Recombinant HLA-G was kindly donated by Dr Nathalie Rouas-Freiss, Hospital St. Louis, Paris, France). The reference serum, which was obtained from a healthy individual, was used as the positive reference control in all determinations of HLA-G levels in subsequent experiments. To ascertain the fidelity of the ELISA test, we determined coefficient of variation (CV percentage = standard deviation/mean × 100) for random high and low sHLA-G serum samples. Each sample was tested 20 times. The value of CV found for the high level serum was 14%, and for the low serum level the CV was 6.9% (data not shown).

Statistical analysis

The data was analyzed by the paired Student *t*-test using the SPSS statistical package. *P*-values of $<0.0.05$ are considered significant.

Results and discussion

Samples of EDTA plasma and serum separated from the same individual and prepared from the same aliquot of

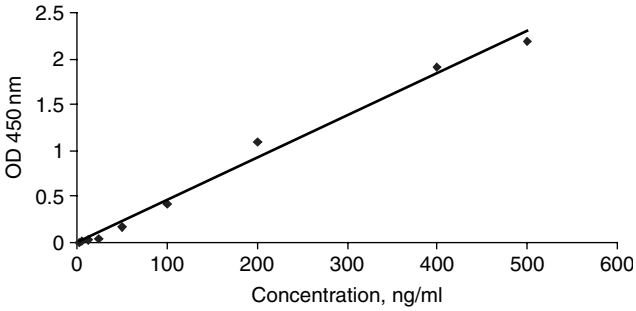

Figure 1 Calibration curve of HLA-G concentration.

blood were tested for the level of HLA-G. All pairs of plasma and serum from a given individual were tested on the same day and on the same ELISA tray. As seen in Table 1 and Figure 2, significant differences were observed between the plasma and the serum samples. In 106 pairs of plasma-serum samples, the average level of HLA-G in the plasma was found to be 38.6 ± 5.0 ng/ml compared with 25 ± 4.6 ng/ml in the serum. These differences are highly significant ($P < 0.001$). To elucidate the reason for this phenomenon, we recalcified EDTA plasma converting the plasma into serum. Table 2 is a summary of representative experiments whereby 19 plasma samples were recalcified by the addition of $CaCl_2$ and thrombine. The level of the plasma HLA-G was determined simultaneously with that of the serum. The level of HLA-G was reduced by clot formation suggesting that HLA-G is either trapped by the clot or consumed during clot formation (plasma level 34 ± 8 ng/ml *vs* recalcified plasma 6.3 ± 3.1 ng/ml; $P < 0.001$). It should be noted that the addition of $CaCl_2$ to serum samples did not reduce the measured values, i.e., $CaCl_2$ by itself has no effect on this level (data not shown). To answer whether HLA-G is trapped or consumed during clot formation, clotted blood was incubated with TPA for 30 min, and the resultant serum was separated form the partially dissolved clot. The results of these experiments are summarized in Table 3. Results suggest that HLA-G is released from the clot, because its level is higher in the solubilized clot compared with the serum. However, levels did not reach initial plasma levels. This can be explained if some of the HLA-G protein is still trapped within the clot and/or consumed.

The interest in soluble MHC antigens was aroused many years ago. As early as 1992, an international workshop on soluble HLA was held with 15 laboratories actively participating in the endeavor (35). In this workshop, many questions were raised including technical and scientific aspects, but the main message was centered on the technical aspects of the procedure. One of the major conclusions was that to reach standardization, the same standard reference antigen, preferably produced by recombinant DNA technology, should be used by all investigators for the creation of a calibration curve.

The interest in the non-classical class-Ib HLA antigens is relatively recent. These antigens probably have important functional significance (19–27, 32). Soluble HLA-G is an important factor in host defense probably via the NK-KIR (KIR2DL4) and myeloid (ILT2/ILT4) receptors. This is, of course, aside from the functions of cell-bound HLA-G expressed on the trophoblast or epithelial thymus (36, 37). In addition, HLA-G was implicated as a possible

Table 1 Levels of sHLA-G in 50 consecutive* plasma and serum samples

Sample	sHLA-G in serum (ng/ml)	sHLA-G in plasma (ng/ml)	Sample	sHLA-G in serum (ng/ml)	sHLA-G in plasma (ng/ml)
1	4	8	336	0	40
3	46	50	337	44	46
4	0	4	339	0	0
6	2	10	344	0	2
7	2	4	345	10	12
11	26	44	347	0	0
12	102	104	356	0	0
15	8	8	357	0	3
18	0	2	359	4	12
20	0	2	363	28	32
21	32	30	366	0	10
23	0	0	368	5	16.6
24	0	2	371	0	8
26	2	2	409	0	138
28	0	4	411	0	110
316	26	30	412	0	32
317	46	46	414	0	210
318	21	46	417	0	44
319	150	164	418	200	210
321	0	32	420	148	140
322	0	18	421	0	29.4
324	80	126	423	150	158
327	0	34	425	12	8
332	30	40	426	72	142
333	0	23.6	428	92	80

*Altogether 106 samples of serum and plasma were studied.

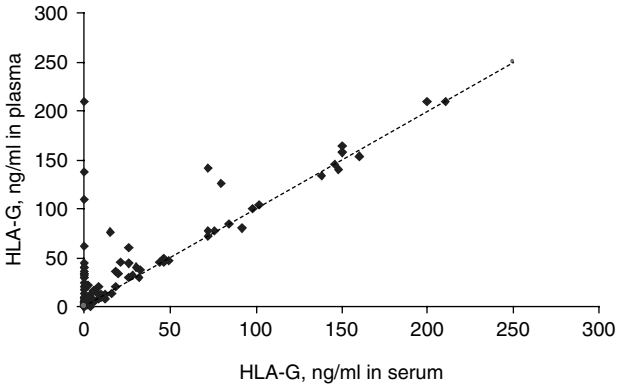

Figure 2 HLA-G levels in serum and plasma. Concentrations of plasma and serum are compared. Samples which are above the oblique line show differences between the serum and plasma levels.

agent by which tumors evade and escape the immune surveillance and enable the development of wide spread malignancy (22, 28–30). HLA-G might also be an important factor in the development of autoimmunity (38). Hence, measurement of the level of HLA-G in the body fluids is an intriguing and important challenge. The reports in the literature measuring the level of HLA-G in the blood are not restricted to either plasma or serum (16, 29, 31–33).

Some investigators have studied serum samples while others measured plasma levels. This creates uncertainty with respect to the true blood level of this protein. In addition, the variations in blood levels are not exclusively the results of measurements of serum or plasma levels, but may also be due to the different protocols of ELISA used by the investigators. Different capturing monoclonal antibodies, incubation times, and washing efficiencies, in addition to differing sensitivities of the substrate and spectrophotometric reading wavelengths may all contribute to substantial differences in the results. For example, Yie et al. (32) found normal serum to be 93 ng/ml while Creput et al. found serum levels to be around 20 ng/ml. Sebti et al. (29) used plasma to test for the levels of HLA-G and found it to be 18 ± 9 ng/ml while Rebmann (16) studied plasma levels and found these were found to be 25 ng/ml in males and 20.1 ng/ml in females. Our results concerning mean plasma level (38.6 ng/ml) are somewhat higher than those reported by Rebmann and Sebti. This may be due to the different standards used for calibration. It supports the argument that an international reference standard, preferably commercially available, should be used by all those measuring the level (serum or plasma) of HLA-G.

Table 2 Levels of HLA G in recalcified plasma samples and original serum

Recalcified plasma levels* (ng/ml)	Plasma levels (ng/ml)	Number of samples
0	0	337
5	52	340
0	44	342
0	32	343
0	20	346
0	4	348
3	14	349
50	130	351
0	42	352
2	10	353
7.2	28.8	354
0	4.9	357
5.6	13.2	359
0	24	360
0	10	363
4.4	72	366
5.6	18.6	368
0	14	371
37.6	108	28,901

*See *Materials and methods*.

Table 3 Levels of HLA G in serum following incubation of clotted blood with TPA

Level following incubation of clotted blood with TPA (ng/ml)	Serum level (ng/ml)	Plasma level (ng/ml)	Number of samples
25.4	0	138	409
2	0	110	411
0	0	32	412
13	0	210	414
4	0	44	417
202	200	210	418
144	148	140	420
0	0	29.4	421
150	150	158	423
8.8	8	12.4	425
122	72	142	426
78	92	80	428
102	100	100	449
0	0	30	451
76	86	62	452
96	86	84	454
16	16	12	455
15.2	18	19.2	457
60	72	72	458
6	0	6	460
22	18	36	461
16	8	20	463
2	0	36	464
0	0	6	467
12	6	8	469

In fact, a recent "wet workshop" was carried out in Essen in 2004 where the participants tested two monoclonal antibodies (MEM G/9 and 5A6G7) and two detecting antibodies, Anti β2m and w6/32 in an ELISA-based technique (39). The investigators reached the conclusion that the technique is reproducible and reliable. The monoclonal antibodies are specific in the determination of soluble HLA-G whether it is G1 or G5 isoforms. However, they did not test different sources of soluble HLA-G such as plasm *vs* serum.

Our experiments address only one of these factors, namely the source of the blood product used for the assay. We show that different values are obtained when plasma or serum is measured. Such variations may lead to erroneous conclusions concerning HLA-G levels in health and disease.

The different levels can be explained if HLA-G is trapped in the blood clot, resulting in reduced serum levels. Reduced serum levels could also arise from decreased stability of HLA-G in the serum compared with plasma. However, the reduction of its level following recalcification of plasma is not a strong support for such a contention. The reduction is inconsistent and varies between individuals.

The lack of standardization creates uncertainty with respect to the biological importance of HLA G. It is therefore suggested that international collaborative studies should be carried out using common commercially available pure HLA-G as the calibration agent and common monoclonal antibodies and plasma as the substrate used for HLA-G determination.

Acknowledgment

This research was supported in part by a grant no. 652/04 to E. Gazit by the Israel Science Foundation (ISF).

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