

Impact of blood processing on estimation of soluble HLA-G

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HLA-G is a non-classical MHC class I antigen that functions as an immunomodulatory molecule. There are two forms of HLA-G antigens, soluble and membrane bound. Soluble HLA-G can be produced by translation of HLA-G transcripts (HLA-G5, -G6, -G7) and by shedding/proteolytic cleavage of membrane bound antigens (HLA-G1, -G2, -G3, -G4). Soluble as well as membrane bound HLA-G molecules have a direct inhibitory effect on immune responses. The relevance of soluble HLA-G in various pathologic conditions, such as transplantation, autoimmunity, infectious and malignant diseases, has been extensively investigated, however interpretation remains controversial.

In this work we analyzed the levels of sHLA-G (sHLA-G1 and HLA-G5) in different blood samples of healthy donors as serum, and blood plasma isolated using anti-coagulant EDTA and heparin, respectively. We found that the levels of sHLA-G (sHLA-G1 and HLA-G5) in blood plasma prepared with EDTA were significantly higher than those observed in plasma with heparin or in serum. Finally we detected the average levels of sHLA-G in females exceeded those of males.

Key words: soluble HLA-G, ELISA, plasma, serum

HLA-G is a human non-classical major histocompatibility class I antigen characterized by a limited polymorphism and restricted tissue distribution. Due to alternative splicing of primary transcript four membrane-bound antigens (HLA-G1, -G2, -G3, -G4) and three secreted soluble molecules (HLA-G5, -G6, -G7) can be produced. Soluble HLA-G antigens (sHLA-G) can be produced not only by translation of HLA-G transcript isoforms (HLA-G5, -G6, -G7) but also by shedding and proteolytic cleavage of membrane bound antigens (HLA-G1, -G2, -G3, -G4) [1-4]. The soluble HLA-G5 and HLA-G6 are directly distinguishable from the other protein isoforms due to the presence of a peptide derived from translating the short part of a retained intron-4 sequence instead of the transmembrane domain and cytoplasmic domain [5, 6]. Like their surface membrane bound counterparts, sHLA-G molecules have immunosuppressive properties [7-9]. Soluble HLA-G antigens have been described thus far in serum/plasma, amniotic fluid, ascites and cerebrospinal fluid. Whether they are also present in urine, tears and milk, like classical HLA class I antigens remains to be determined [10].

A significant amount of information has been gathered about the functional characteristics of sHLA-G and its potential clinical relevance. It has been demonstrated that serum/plasma sHLA-G levels can be changed at various

physiological and pathological conditions as pregnancy, organ transplantation, autoimmune disease, viral infection and especially at malignant diseases [11, 12]. Hunt et al. proved that serum levels of sHLA-G were significantly higher in pregnant women than in nonpregnant individuals [13]. However HLA-G level in plasma from women who subsequently developed preeclampsia disorder was lower than in control patients [14]. It was detected that serum levels of sHLA-G were increased at some diseases of autoimmune origin e.g. multiple sclerosis [15]. High level of plasma sHLA-G correlated with better graft acceptance of patients who underwent transplantation of heart, kidney and liver, respectively [16-18]. Soluble HLA-G antigens have been detected in plasma/sera of patients with various types of malignant diseases. For example, ovarian serous carcinoma and ductal breast carcinoma, are associated with high amount of soluble HLA-G in tumor ascites [19]. A significant high level of sHLA-G was observed in plasma/serum of patients with melanoma [20], lymphoproliferative disorders (B-CLL, T-NHL, B-NHL and AML) [21, 22], neuroblastomas [23] and with carcinomas of various organs as lung, breast, colon, liver, kidney, etc. [24-29]. Thus, HLA-G is becoming a relevant marker in various pathologies, a methodology to precisely measure soluble HLA-G levels is increasingly in demand.

The availability of standardized reagents (particularly monoclonal antibodies) and assays has eliminated to a great extent the variability of the results obtained in different laboratories. The most important procedure used for estimation of sHLA-G in biological fluids is sandwich ELISA [30-32]. Although this method was validated in 2004 (at the "Wet-workshop for quantification of sHLA-G" in Essen), it needs further standardization steps, most importantly, rules/guidelines for preparation of biological samples. For detection of sHLA-G in blood either serum or plasma can be used. Surprisingly significantly different values for sHLA-G were observed between EDTA plasma and serum samples [33].

In this work sHLA-G was evaluated in sera and plasma of healthy donors, however plasma samples were prepared using two different anti-coagulants (EDTA or heparin). Data from EDTA plasma were significantly higher than in plasma prepared with heparin. However, similar low values of sHLA-G in heparin plasma were detected also in serum samples.

Materials and methods

Blood samples. Peripheral blood samples were obtained after informed consent of all healthy volunteers. Serum was separated from clotted blood by centrifugation. Plasma was prepared from blood collected into tubes with anti-coagulants as potassium (K₂) EDTA or lithium heparin. All collected blood samples (serum: S1-S26; plasma-EDTA: E1-E41; plasma-heparin: H1-H26) were stored before use at -70°C.

Antibodies. In this work the following mouse monoclonal antibodies (mAb) were used: MEM-G/9 and biotinylated W6/32. Both mAb were purchased from EXBIO (Prague, Czech Republic). MEM-G/9 is specific for HLA-G molecules associated with β 2-microglobulin. It recognizes not only membrane-bound HLA-G1 isoform, but also its soluble counterpart sHLA-G1 and the secreted isoform HLA-G5. W6/32 antibody binds to all intact HLA class I antigens (in complex with β 2-microglobulin). MEM-G/9 was applied as a capture mAb in sandwich ELISA and biotinylated W6/32 as a detection mAb.

Cells. The lymphoblastoid cell line LCL-721.221 and its HLA-G5 transfectant LCL-721.221-G5 were cultured in RPMI 1640 medium supplemented with 2mM L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), amphotericin B (0.125 μ g/ml) and 10% heat-inactivated fetal bovine serum. The transfectant cells were maintained in medium containing geneticin (1 mg/ml).

Medium from cultured cells LCL-721.221 was used as a negative control and from transfectant LCL-721.221-G5 as a positive control. The concentration of soluble HLA-G in supernatant of cultured HLA-G5 transfectant was standardized using sHLA-G ELISA assay kit (Exbio, Prague, Czech Republic).

ELISA. The sandwich ELISA was used to detect sHLA-G antigens (sHLA-G1 + HLA-G5) in blood samples (serum, plasma-EDTA and plasma-heparin) of healthy donors.

96 well plates (Sarstadt or Greiner Bio-One 96 well plate MICROLON® 200) were used. After coating wells with 50 μ l of mAb MEM-G/9 (100x diluted in carbonate buffer pH 9.6) the plates were incubated overnight at room temperature and then four times washed with 0.05% Tween20 in PBS (PBS-T). Subsequently blocking was performed with 3% BSA in PBS for 1 hour at 27°C. After washing (three times) with PBS-T, 50 μ l of healthy donor blood sample (plasma-EDTA, plasma-heparin, and serum, respectively) was added to each well. The plates were then incubated for 90 min at 27°C and subsequently washed 3 times with PBS-T. After adding 50 μ l of biotinylated mAb W6/32 (400x diluted in 1% BSA in PBS) the plates were incubated for 90 min at 27°C. The wells were then washed 3 times with PBS-T and 50 μ l of streptavidine-HRP (ImmunoPure® Streptavidin, horseradish peroxidase conjugated, Pierce 1mg/ml) diluted 1/1000 in 1% BSA in PBS was added. The next incubation was performed at 27°C for 1 hour. Following washing (3 times with PBS-T and once with PBS), 50 μ l of HRP-substrate was added. In these experiments we used two different HRP-substrates (both from Thermo Scientific, Pierce), either 1-Step™ Ultra TMB-ELISA substrate or SuperSignal ELISA Femto Luminol. In the case of substrate TMB (3,3',5,5'-tetramethylbenzidine), the plates were incubated at room temperature for 15 min and the enzymatic reaction was stopped after adding 50 μ l of 2 M sulfuric acid. The absorbancy was measured at 450 nm using Labsystems Multiskan^R Multisoft microplate reader (Labsystems, Helsinki, Finland). If chemiluminiscent reagent Luminol was used, reaction was terminated with 50 μ l of 1M sulfuric acid and immediately measured in luminometer LUMISstar GALAXY (BMG Labtechnologies GmbH, Germany). The results were evaluated in relative light units (RLU). All plasma and sera samples were tested in duplicates.

Statistical analysis. The distributions of sHLA-G levels among three different groups of peripheral blood samples (serum, plasma-EDTA, plasma-heparin) were characterized through median and interquartile range. Comparison of sHLA-G levels was analyzed by two-sided Student t test. A probability of <0.05 was considered statistically significant.

Results and discussion

Level of sHLA-G in blood is increased in a number of pathological disorders, including malignant diseases. Often there is a good correlation between the level of sHLA-G with clinical stage and tumor grade, allowing better characterization of disease/therapy progress. Plasma or serum is used to determine sHLA-G in blood. Blood plasma is prepared by using anti-coagulant heparin or EDTA. Heparin works by activating antithrombin III, which blocks thrombin from clotting blood. EDTA removes from fresh blood calcium ions that prevents formation of thrombin. Blood serum is blood plasma without fibrinogen and other clotting factors.

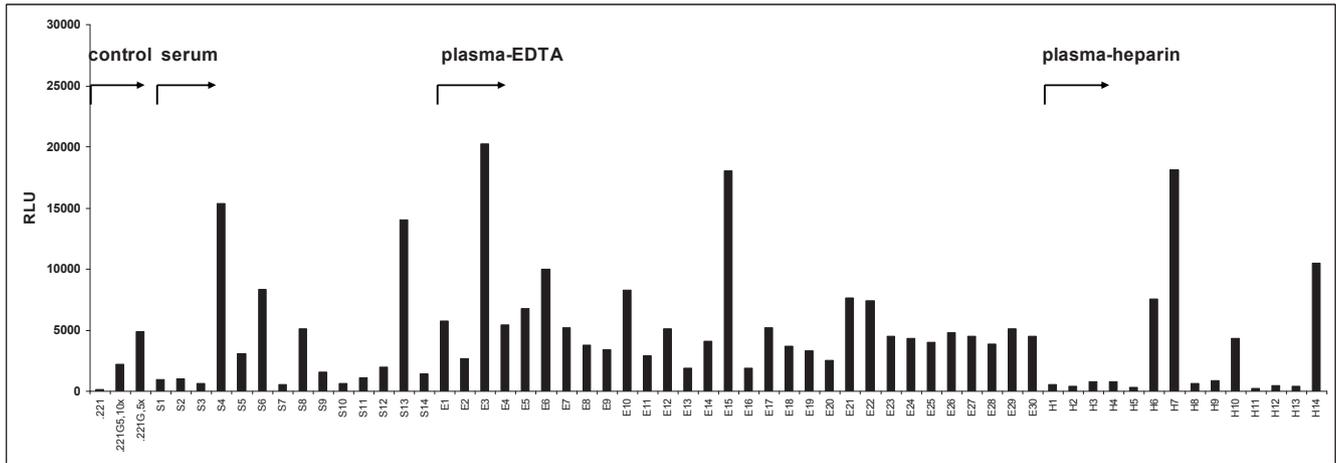


Figure 1. sHLA-G levels in peripheral blood samples of healthy donors determined by ELISA (with luminol). Blood samples: serum (S1-S14), plasma-EDTA (E1-E30), plasma-heparin (H1-H14). Controls: .221 (negative control – medium from cultured cells LCL-721.221); .221G5 (positive control – medium from cultured transfectant LCL-721.221-G5 was diluted 5x or 10x). Chemiluminiscent reagent luminol was used as a substrate and sHLA-G was estimated in relative light units (RLU).

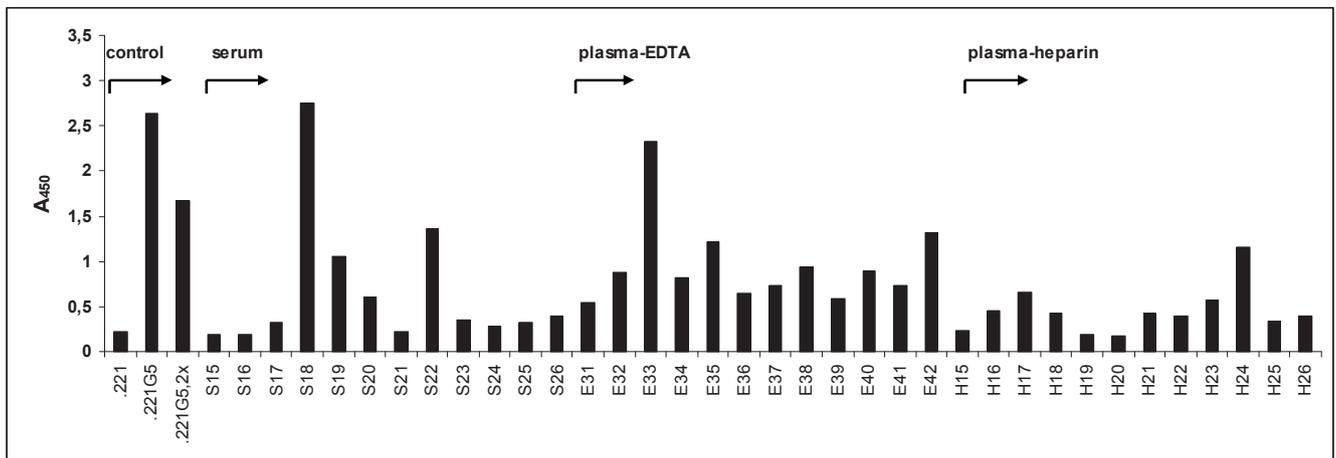


Figure 2. sHLA-G levels in peripheral blood samples of healthy donors determined by ELISA (with TMB). Blood samples of healthy donors: serum (S15-S26), plasma-EDTA (E31-E42), plasma-heparin (H15-H26). Controls: .221 (negative control – medium from cultured cells LCL-721.221); .221G5 (positive control – medium from cultured transfectant LCL-721.221-G5 was undiluted, or diluted 2x). Ultra TMB reagent was used as a substrate and sHLA-G was estimated as absorbance at 450 nm.

Rudstein-Svetlicky et al. comparing sHLA-G levels in blood samples of healthy individuals found that HLA-G values in plasma with EDTA were higher than in serum samples [33]. We decided to extend this study and to assess sHLA-G in blood samples including also heparin plasma. Medium prepared from cultured HLA-G5 transfectant (LCL-721.221-G5) was used as a reference sHLA-G positive control. The concentration of sHLA-G in such reference sample was estimated by calibration with standard obtained from sHLA-G specific ELISA kit (Exbio, Prague, Czech Republic). All three types of blood samples (serum, plasma-EDTA and plasma-heparin)

were divided into two groups and analyzed by sandwich ELISA with two different substrates for HRP: luminol and TMB, respectively (Fig.1 and Fig.2). As seen in box plot analysis, in both ELISA assays the significantly higher level of sHLA-G was detected in plasma-EDTA than in serum (Fig.3). This is in agreement with the results published by Rudstein-Svetlicky et al. [33], who suggested that some sHLA-G in serum is trapped during clot formation. As the amount trapped within the clot is variable it is very difficult to obtain values reflecting true biological levels of sHLA-G in blood. Surprisingly we found the median level of sHLA-G in serum and in heparin plasma

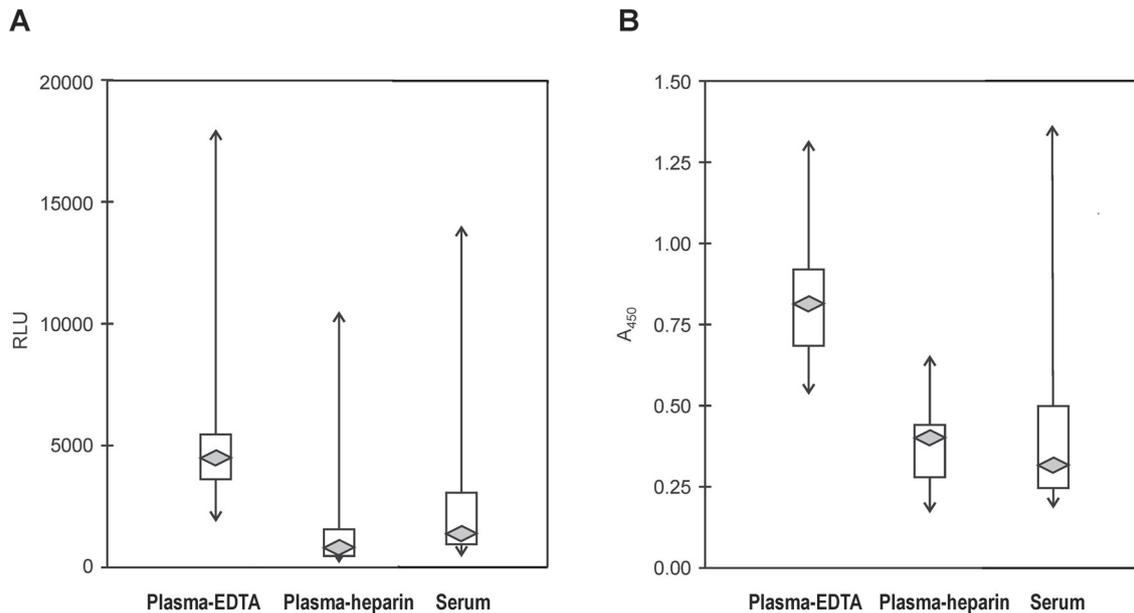


Figure 3. Impact of blood sampling on determination of sHLA-G.

A: sHLA-G levels determined by ELISA (with luminol). Blood samples: serum (S1-S14), plasma-EDTA (E1-E30), plasma-heparin (H1-H14). Plasma-EDTA vs. plasma-heparin: $p=0.0009$; plasma-EDTA vs. serum: $p=0.0740$.

B: sHLA-G levels determined by ELISA (with TMB). Blood samples: serum (S15-S26), plasma-EDTA (E31-E42), plasma-heparin (H15-H26). Plasma-EDTA vs. plasma-heparin: $p<0.0001$; plasma-EDTA vs. serum: $p=0.0160$.

Arrows indicate range of values; column contains 50% of values grouped around median marked with diamond.

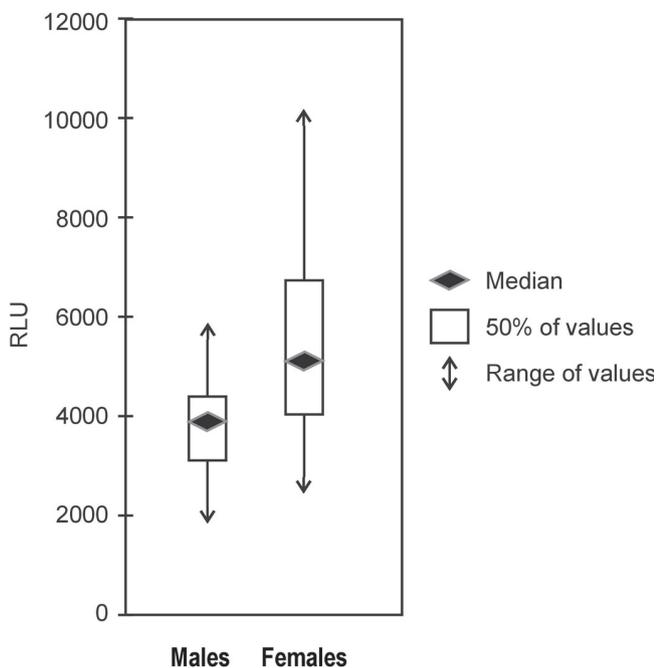


Figure 4. Gender difference in sHLA-G levels of healthy donors. sHLA-G was determined in plasma-EDTA samples (E1-E30) using ELISA with luminol. Arrows indicate range of values; column contains 50% of values grouped around median marked with diamond. Males vs. Females: $p=0.0198$.

almost identical (Fig.3A and Fig.3B). Adding heparin to blood does not cause any clot formation, so theoretically no sHLA-G can escape detection, and no difference between EDTA and heparin plasma was expected. So far we have no explanation why sHLA-G estimated in EDTA plasma and heparin plasma is different.

Quantitative determination of sHLA-G in body fluids needs not only appropriate sample preparation but also a well-defined sHLA-G protein standard. Unfortunately no commercial sHLA-G standard is available and the Exbio sHLA-G kit allows determining only the units of sHLA-G (U/ml). Non standard sample preparation and lack of reliable standard almost certainly results in extremely different data on sHLA-G concentration in plasma of healthy subjects. The mean levels of sHLA-G in plasma of healthy subjects estimated by others ranged from 20 ng/ml to 400 ng/ml [26, 34].

It was reported that in some populations, there is a gender difference in level of sHLA-G in blood of healthy individuals. The results are controversial. Either it was reported that the average levels of HLA-G in males exceeded those of females or that there is no difference. Therefore we decided to determine levels of sHLA-G in plasma-EDTA from 18 healthy females and 12 males. Our results clearly show the average levels of sHLA-G in females exceeded those of males (Fig.5). The reason for the gender differences in levels of sHLA-G is unknown. One apparent possibility is that hormones (e.g. progesterone) may

influence the level of sHLA-G in blood [35], nevertheless to explain this difference further examination is required.

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