—Mini Review—

Can Soluble HLA-G Protein Be a Marker for the Selection of IVF Embryos?

Akiko Ishitani[1]*, Noriko Sageshima[2],
Mari Nakanishi[1] and Katsuhiko Hatake[1]

[1]*Department of Legal Medicine,*
[2]*Department of Bacteriology, Nara Medical University, 840 Shijocho, Kashihara,
Nara 634-8521, Japan*

Abstract: *Soluble HLA-G (sHLA-G) has been reported as a biomarker for embryo quality in human in vitro fertilization (IVF). The first report showed that some embryos had secreted sHLA-G and that the secretion was necessary for implantation. If these data are true, sHLA-G could be a very useful marker for embryo selection. However, one major limitation of the report was a distinct lack of controls relevant to the methods of measurement. Therefore, we re-examined the detection of sHLA-G in 109 culture supernatants from embryos fertilized in vitro. We were unable to detect sHLA-G in any supernatant despite using a more sensitive method than that used in the previous report. We further explored other reports that had shown positive data of sHLA-G secretion from embryos, and found that neither standard curves to enable calculation of sHLA-G concentration correctly nor any data for calibration were available in these reports. In this review, we point out several problems with the detection of sHLA-G in the previous reports, and describe controls and methods that can be used to determine sHLA-G concentrations accurately.*
Key words: *Soluble HLA-G, Embryo, ELISA*

Introduction

The human major histocompatibility complex (MHC) class I genes, HLA-E, -F, -G, which were discovered by Geraghty and colleagues in 1987–1990 [1–3], are referred to as non-classical or class Ib genes, and are located on the telomere side of the class I region of chromosome 6 (Fig. 1). They are distinguished from their close relatives, the classical class I genes, by expression patterns and by low allelic polymorphism. After their discovery, HLA-G was the most exclusively studied of the three HLAs, and several aspects of its expression and possible function were soon elucidated. The expression of HLA-G protein is primarily restricted to placental trophoblasts, and soluble and membrane forms of the protein are expressed as derivatives from alternative splicing of the primary mRNA [4–7]. However, several papers have reported HLA-G expression in different tissues or fluid, for example, tumor tissues or lymphocytes after transplants [8, 9]. These sites of expression are still being debated and some results have not yet been replicated. The function of HLA-G is comparatively well elucidated. HLA-G protein has been shown to associate with leukocyte immunoglobulin-like receptor (LILR)-B1 (also known as immunoglobulin-like transcript (ILT)-2) [10] expressed on lymphocytes, LILR-B2 (also known as ILT-4) [11] expressed on myeloid cells, and killer-inhibitory receptor, KIR2DL4 [12], expressed on natural killer (NK) cells, although the latter interaction has not been confirmed. HLA-G is hypothesized to inhibit the activity of immune cells through these inhibitory receptors. Hence, in addition to its roles in the reproductive immunological system, it has been suggested that HLA-G plays a role in tumor immunity and transplantation immunity. One important function of HLA-G may be to provide a nonamer derived from its signal peptide to HLA-E in the placental trophoblasts.

HLA-E, instead of binding common peptides derived from intracellular proteins, is able to bind only nonamer peptides derived from other class I signal sequences as a requirement for cell surface expression [13, 14]. The complex formed of HLA-E that binds to peptide derived from HLA-G was shown to activate NK cells through
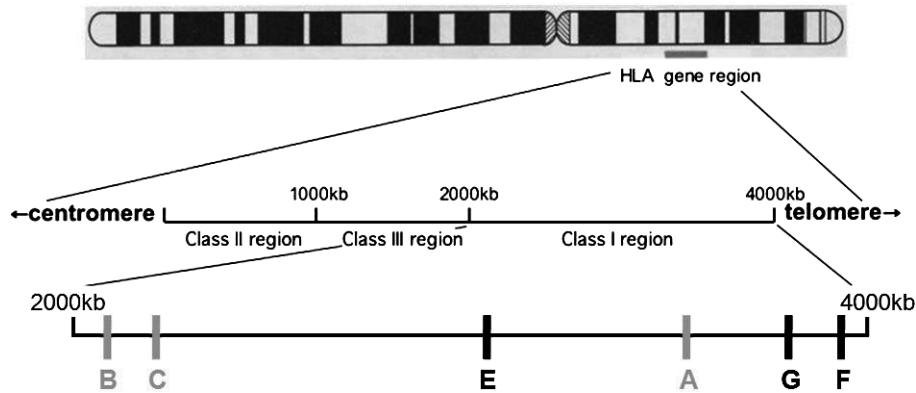
Chromosome 6

Fig. 1.   HLA-E, -F, -G genes are located on the telomere side of the class I region of chromosome 6.

CD94/NKG2C in addition to the interaction of the HLA-E bound peptide derived from classical class I inhibiting NK cells through CD94/NKG2A. Therefore, HLA-E is thought to play an important role in pregnancy.

The study of HLA-F has just started, and only a handful of reports studying its protein expression have appeared. Though HLA-F was considered to be absent from the cell surface [15], we found its expression on extravillous trophoblasts in term placenta [16, 17]. Although the function of HLA-F is not understood yet, it is clear that HLA-F may have an important role in pregnancy, because it is expressed only by trophoblasts among normal human tissues.

The interaction of all three non-classical class I molecules may be an important overall role for pregnancy rather than the role of each molecule individually. Recently, HLA-G has received much attention as an immunosuppressive molecule that inhibits NK cells or induces apoptosis of CD8+ T cells [18, 19]. Considering these studies, HLA-G might be secreted from early embryos to inhibit maternal immune cells that are cytotoxic to the embryo, thereby facilitating a successful implant.

In 2002, Fuzzi *et al.* reported that soluble HLA-G (sHLA-G) was detected in culture medium of *in vitro* fertilized eggs, and only the sHLA-G secreting embryos implanted successfully [20]. If this were true, HLA-G could be an useful tool for identifying good-quality embryos. One major limitation of the report of Fuzzi *et al.* was that it provided no precise description of the method or the standard curve to determine sHLA-G

concentration. After the report of Fuzzi *et al.*, a number of similar studies with similar shortcomings have been published [21–25], even though we had pointed out the problems with the method [26]. In these publications, it was not clear how the concentrations of sHLA-G protein in culture media were determined. In contrast, one report by Van Lierop *et al.* observed no production of sHLA-G in fertilized embryos [27]. Their study presented a standard curve and described the method of purifying sHLA-G protein used to produce that standard curve. The inconsistency among these studies might be the result of using different ELISA methods and the lack of a standard curve to estimate protein concentration. Therefore, we re-examined the measurement of sHLA-G using a sensitive and specific ELISA method that we developed to clarify the contradictions [28].

Re-examination of sHLA-G in IVF Culture Supernatant

We tested 109 culture supernatants from day 2–3 embryos (84 samples) and day 4–6 embryos (25 samples) fertilized by conventional IVF method or intracytoplasmic sperm injection (ICSI). After fertilization, embryos were cultured in HTF (W.A.COOK, Australia) containing 10% serum substitute supplement (SSS) (Irvine Scientific, CA, USA) or Cleavage Medium (W.A.COOK, Australia) for 3 days, and then in Blastocyst Medium (W.A.COOK, Australia) for 1-3 additional days. Some of the day 4–6 embryos had
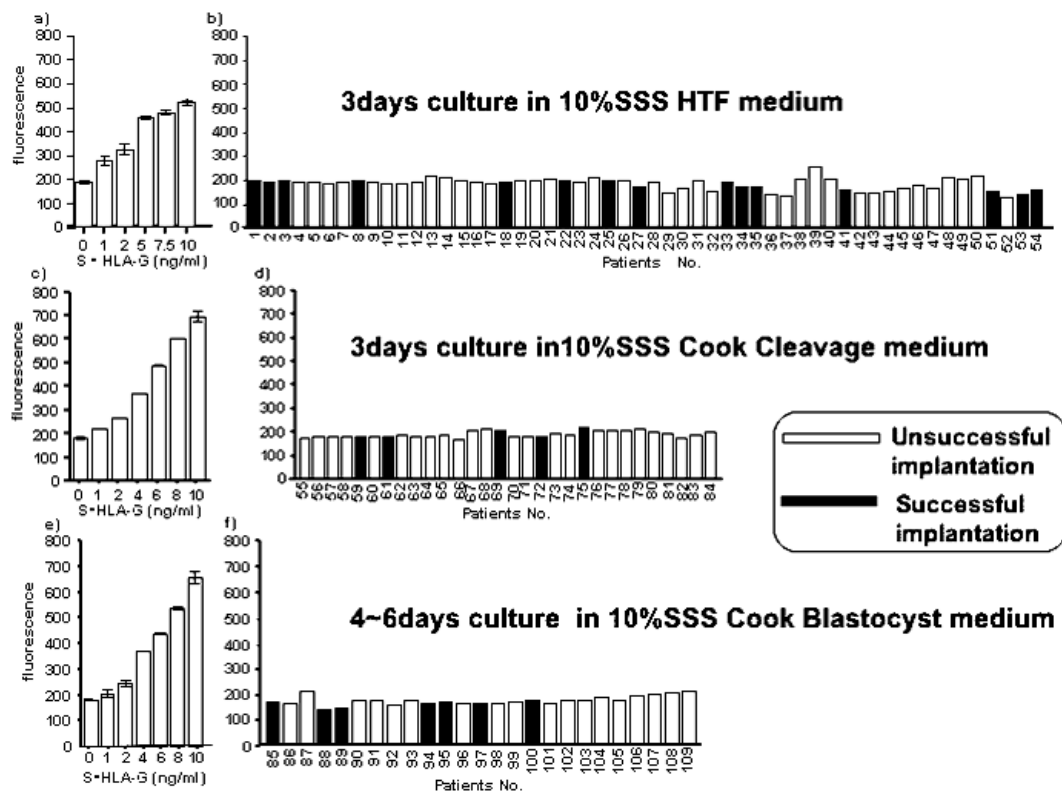
Fig. 2. Detection of sHLA-G protein in the culture supernatants of fertilized eggs. (a, c and e) The standard curves of sHLA-G dissolved with 10%SSS-HTF, 10%SSS-COOK Cleavage and 10%SSS-COOK Blastocyst, respectively. The fluorescence intensity is shown on the verticcal axis and the concentration of sHLA-G is shown on the horizontal axis in a, c and e. Data are presented as the mean ± SD. Panel b shows the results of 54 supernatants of 2–3 day embryos in 10%SSS-HTF. Panel d shows the results of 30 supernatants of 2–3 day embryos in 10%SSS-COOK Cleavage. Panel f shows the results of 25 supernatants of 4–6 day embryos in 10%SSS-COOK Blastocyst. The fluorescence intensity is shown on the vertical axis and the number of supernatants on the horizontal axis in b, d and f. In b, d and f, the fluorescence intensity of the supernatant resulting from successfully implanted eggs is shown as a black bar, and the fluorescence intensity of the supernatant resulting from unsuccessfully implanted eggs is shown as a white bar.

reached the blastocyst stage. For the determination of sHLA-G, a sandwich ELISA method with anti-HLA-G monoclonal antibody, MEM/G9 (EXBIO Praha, Czech Republic), for capture, and anti-HLA-class I antibody, W6/32, for detection were used. These reactions were detected by UV fluorescence which is more sensitive than visible ray. The standard curves were made using affinity purified sHLA-G from medium conditioned by 721.221-Gs cells (.221-Gs), a B-lymphoid cell line, 721.221(.221), transfected with sHLA-G cDNA [29].

The results indicated that there was no sHLA-G detectable by ELISA in any sample (Fig. 2). The variations of the fluorescence intensity of the samples were within the deviation of background. Therefore, we concluded that sHLA-G in culture medium is not a

useful marker for successful implantation at this stage of development.

Comparison of sHLA-G Detection Methods

Our results raised the question of how Fuzzi *et al.* detected sHLA-G. To try to reconcile these results, we compared the ELISA method and the calibration curve between our method and Fuzzi's (Table 1 and Fig. 3). The standard curve of Fuzzi's method was constructed using the method described in their report since they did not show any calibration curve. To examine the specificity of the method, anti HLA-E antibody, 3D12, and isotype control, mouse IgG1, were used for the capture antibody. In our methods, the fluorescence

Table 1. Comparison of the ELISA methods, Sageshima *et al.* and Fuzzi *et al.*

	Sageshima *et al.* (2007)	Fuzzi *et al.* (2002)
Pretreatment of sample	Centrifugation at 12,000 rpm at 4°C for 20 min	Not described
Incubation time for coating with MEMG/9	At 4°C for 1 night (20 μg/ml)	At 37°C for 1 h (20 μg/ml)
Blocking	2%BSA-PBS at 37°C for 2 h	4%BSA-PBS at 4°C for 1 night
Standard protein	Affinity-purified sHLA-G from culture supernatants of .221-Gs	Culture supernatant of sHLA-G/.221-Gs (not affinity-purified)
Negative control	Media used for embryo culture	Not described
Incubation time for the sample	At 4°C for 1 night	At 37°C for 2 h
Incubation time for detection with biotinylated W6/32	At 25°C for 1 h (20 μg/ml)	At 37°C for 1 h (concentration; not described)
Detection	β-D-galactosidase Fluorescence (4-methyl umbelliferone; 330 nm)	Peroxidase Coloring (o-phenylenediamine; 405 nm)
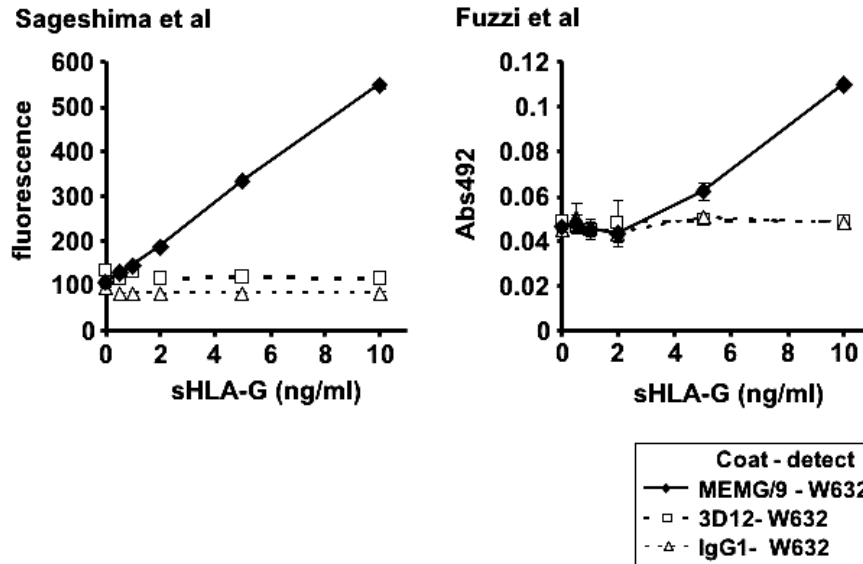
Fig. 3. A comparison of the sensitivity for detecting soluble HLA-G between alternative ELISA methodologies. We performed the ELISA according to the method described Sageshima *et al.* (A) and that published by Fuzzi *et al.* (Fuzzi *et al.*, 2002; Fournel *et al.*, 2000b) (B). Three different monoclonal antibodies were tested for the capture antibody, MEMG/9 (anti HLA-G), 3D12 (anti HLA-E) and mouse IgG1 (isotype control) as described in the methods. The results of MEMG/9-biotinylated W6/32 (●), 3D12-biotinylated W6/32 (■) and mouse IgG1-biotinylated W6/32 (▲) are shown. The values are presented as the mean ± SD. The fluorescence intensity or absorbance is shown on the vertical axis and the concentration of sHLA-G is shown on the horizontal axis in A and B.

intensity increased linearly with the concentration of sHLA-G (Fig. 3A). On the other hand, in Fuzzi's method, the absorbance intensity did not increase linearly at concentrations of HLA-G from 0 to 2 ng/ml (Fig. 3B). Though Fuzzi *et al.* insisted that they could detect 1.4 ng/ml of sHLA-G protein, our re-examination

revealed that Fuzzi's method is not accurate below 2 ng/ml of sHLA-G protein. The increase in the fluorescence intensity ranging from 0 ng/ml (media) to 10 ng/ml was from about 100 to 600, 5 times higher than background, using our method. On the other hand, the increase in the absorbance of sHLA-G ranging from

0 ng/ml to 10 ng/ml was from 0.04 to 0.12, only 2 folds greater than the background with Fuzzi's method. This result indicates that our method is more sensitive at detecting sHLA-G in body fluids. Although we could not detect sHLA-G in the culture supernatants using our sensitive method, the implantation rate of day 2–3 embryos was 23% and of day 4–6 embryos was 31%, while it was only 24% in the sHLA-G positive embryos described in Fuzzi's report. Therefore, we concluded that sHLA-G was not secreted from the embryo and is unsuitable as a marker for selecting good quality embryos.

Problems in Recent Reports

In 2006, Desai *et al.* [30] reported that they tested the culture supernatant of 712 embryos from 83 patients, and detected sHLA-G in 306 samples using sHLA-G ELISA Kit (EXBIO Praha, Czech Republic). They described that the concentration of sHLA-G in day 3 embryo culture media ranged from 3 to 50 ng/ml, and that the majority of samples had low concentrations (3–10 ng/ml), whereas high concentrations (>10 ng/ml) were detected in only 14% of samples. They also reported that the sHLA-G concentration in culture supernatants had no association with therapeutic outcomes. Although they did not show the standard curve to determine the concentration, it is available in the manufacturer's instructions (Fig. 4). However, using this standard curve, it seems difficult to determine an accurate concentration in the range of 3–10 ng/ml, the range of the majority of positive samples described by Desai et al. In 2007, Rizzo *et al.* [31] reported the detection of sHLA-G in culture supernatants of fertilized oocyte (FO) and follicular fluid (FF). They claimed that 19/50 (38%) of FFs and 26/50 (52%) of FOs were sHLA-G positive, and that 18 sHLA-G positive supernatants corresponded to the 19 sHLA-G positive FFs (94.7%), whereas the other 8 sHLA-G positive supernatants correspond to the 31 sHLA-G negative FFs (25.8%) showing a relationship in sHLA-G production between FF and oocyte supernatants. Although they also did not show the standard curve to determine sHLA-G concentration, it is possible to surmise the accuracy from the standard curve reported at the Essen Workshop on sHLA-G quantification (Rebmann *et al.* 2005) since this method was used in their study. They reported that sHLA-G was detected in 26 (52%) oocyte cultures, and the median was 4.35 ng/ml (ranging from 1.2–13.1 ng/ml). From the standard curve shown in Fig. 5, it is difficult to determine a
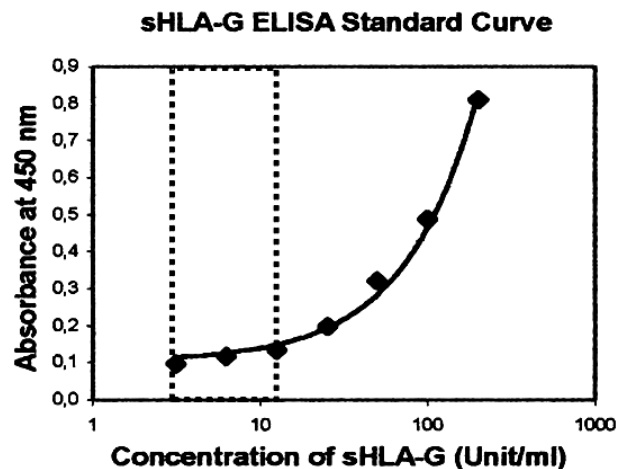
Fig. 4. Soluble HLA-G ELISA standard curve presented by the manufacturer (EXBIO Praha, Czech Republic) for the calculation of the concentration using their sHLA-G ELISA Kit. The manufacturer state that the sHLA-G protein used as the standard was not pure and that 1 unit corresponded to approximately 0.5 ng of protein.
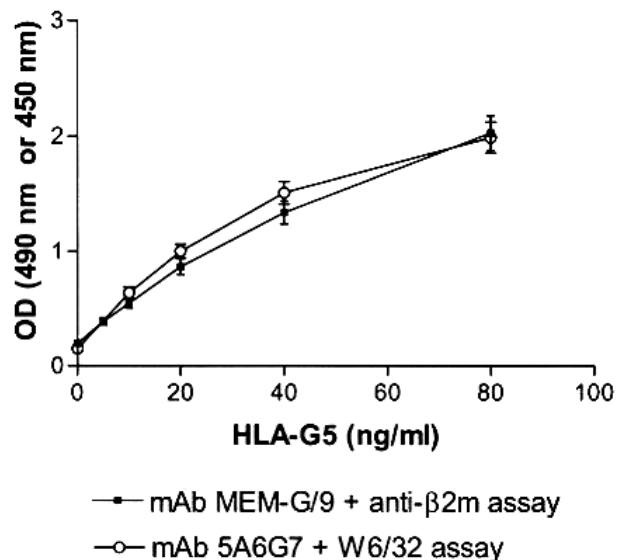

Fig. 5. Soluble HLA-G standard curve of ELISA methods using either monoclonal antibody MEM-G/9 + anti-human β2m or 5A6G7 + w6/32 as antibody combinations according to the method used at the Wet Workshop for Quantification of Soluble HLA-G in Essen, 2004. Each point represents the mean ± SEM value obtained by the different workshop teams (n=11). (From Rebmann *et al.* 2005. Hum. Immunol.)
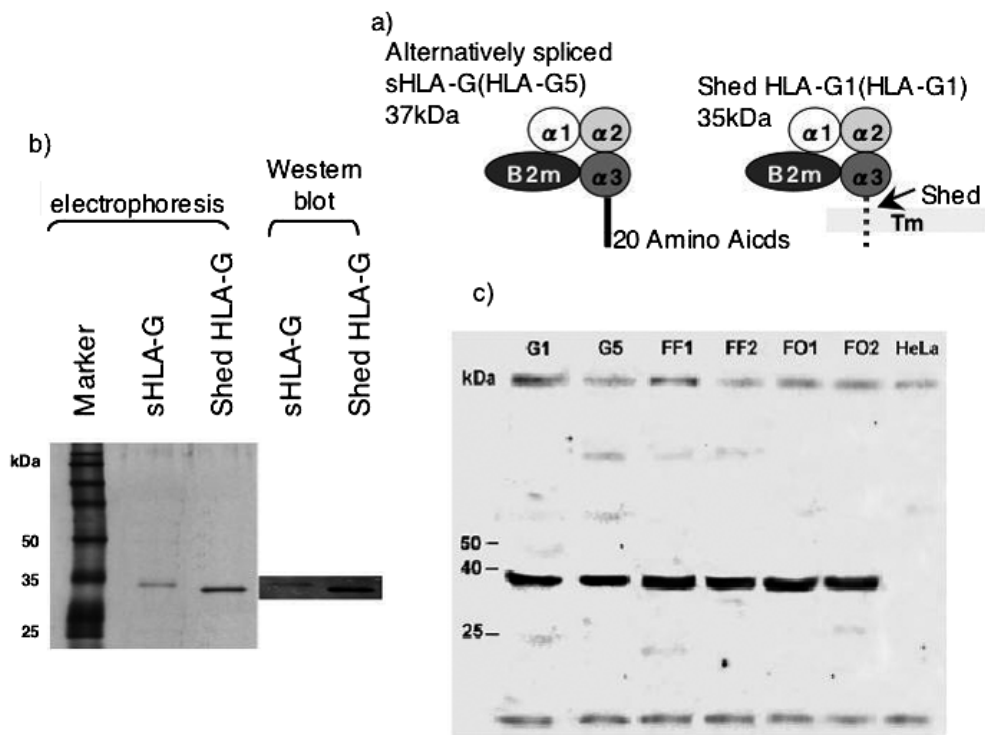
Fig. 6. Western blot analysis of two distinct sHLA-G proteins with different molecular weights probed with MEM-G1 antibody. a) The image shows two kinds of soluble HLA-G molecular structures, an alternatively spliced sHLAG1 form which has a 20 amino acid tail and HLA-G1 shed from cell membrane which has no tail. b) The western blot tested sHLA-G purified from culture supernatant from the 221-Gs transfectant (alternative isoform, sHLA-G) and shed type HLA-G1, and showed a clear difference in size between the two proteins. c) Western blot by Rizzo *et al.* [31] for 10× follicular fluid (FF), 100× oocyte culture supernatant (FO) and culture supernatant of transfectant 221-G1 and Hela-G5. All proteins appear as the same size despite the authors claim that FF included 2 distinct forms of sHLA-G.

concentration of 4 ng/ml, since the difference of OD at this concentration is only 0.1 units over the 0.2 units of the background. Moreover, sHLA-G positive FF included two different forms of sHLA-G1, HLA-G1 and HJLA-G5. The former is produced by proteolytic cleavage or shed from membrane-bound HLA-G, and the latter is produced from alternatively spliced mRNA. Oocyte supernatant (FO) included only sHLA-G1. These two HLA-Gs are easily distinguishable by gel analysis, as the size of HLA-G5, which has 20 amino acid tails at the carboxy terminal, is 37 kDa and that of sHLA-G1, which has no tail, is 35 kDa (Fig. 6a, 6b). However, western blot analysis of ×10-concentrated FFs and ×100-concentrated oocyte supernatant (FO) appeared as the same molecular size despite the authors' claim that FF included 5 distinct forms of sHLA-G (Fig. 6c).

Almost all of the papers that reported positive data for sHLA-G secretion from embryos did not show the standard curve with precise methodology, except for the latest study by Rebmann *et al.* [32], who reported that morphologically good embryos secreted 0.6 ng/ml and morphologically bad embryos secreted 0.1 ng/ml using the standard curve shown Fig. 7. However, this standard curve appears inconsistent with the ability to determine such low concentrations accurately.

Contamination of Class I HLA Proteins

Our results, in which sHLA-G was not detected in embryo culture at any levels above background, led us to investigate other confounding issues that might be responsible for false positive results. Although the misinterpretation of low concentrations of sHLA-G may result from the inappropriate use of standard curves, it is difficult to explain the reason why high concentrations of
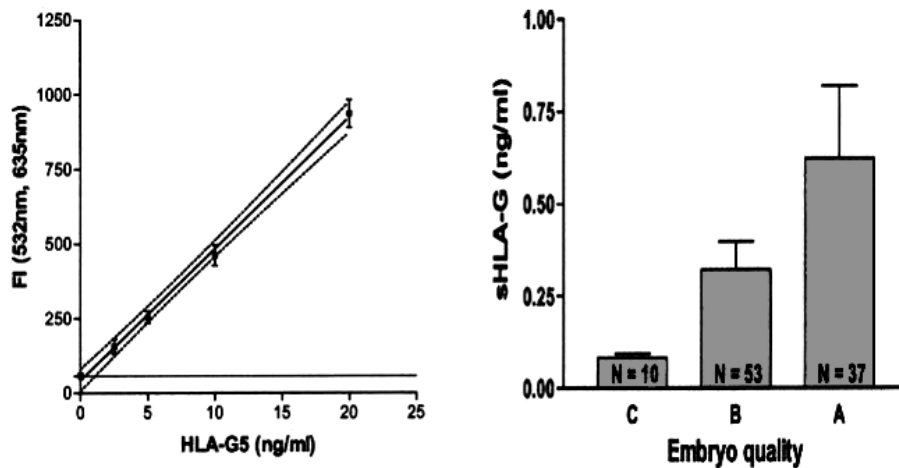
Rebmann V et al. *Human Immunol.* 2007

Fig. 7. Soluble HLA-G standard curve and the results reported by Rebmann *et al.* [32]. There were no clear differences in the ELISA signals between the two concentrations of sHLA-G secreted by embryos that were claimed to have an association with bad (0.1 ng/ml) and good (0.7 ng/ml) embryos.
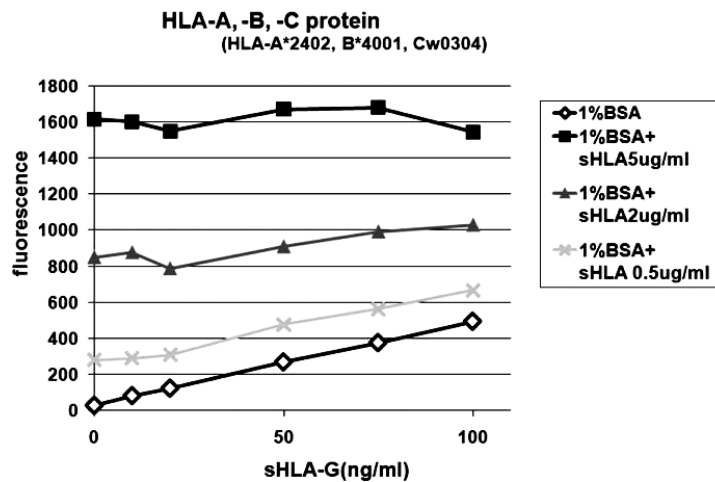

Fig. 8. The effect of HLA classical class I protein on sHLA-G ELISA. Using affinity purified sHLA-G protein from 221-Gs transfectant and affinity purified classical class I protein from HLA homozygous cell lines, increasing signal in the sHLA-G standard curves can be seen with increasing amounts of classical class I protein added.

sHLA-G were detected in some studies. We therefore tested the effect of classical class I HLA protein on sHLA-G ELISA. Though such proteins may not exist in embryo culture supernatants at the levels we tested, it was clear that contaminating classical class I HLA protein affected the ELISA system remarkably (Fig. 8).

Conclusion

A number of papers have insisted that sHLA-G was secreted from some embryos fertilized *in vitro* and that the presence of sHLA-G in embryo cultures was associated with clinical pregnancies. However, most of them lacked appropriate standard curves to determine

low concentrations of sHLA-G. At this point, we suggest that it is indispensable to develop a more sensitive and accurate method to detect sHLA-G protein and to test its accuracy thoroughly before drawing any conclusions about sHLA-G secretion from embryos.

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