Soluble HLA-G, the discussion is going on!

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Introduction

In 1986, Sir Karl Popper explained that refuting a given hypothesis is the only way to achieve progress in science (Popper, 1968). Hence we assume our well-controlled study on intron 4-containing isoforms of HLA-G in human placenta (Blaschitz \textit{et al}., 2005, current issue) was important as it challenged a popular hypothesis and therefore has attracted great attention among the scientific HLA-G-researching community.

We appreciate the initiation of a discussion forum, which was excellently introduced by R. Ivell (current issue). His editorial exactly represents our attitude towards scientific work in general and underlines some of the basic problems of biomedical research nowadays.

The very comprehensive review by Sargent (2005, current issue) summarizes debatable points in the topic of soluble HLA-G isof orm expression and gives a proper overview also to those scientists who are less familiar with HLA-G.

To achieve agreement, special conferences on HLA-G, including pre-workshops, have already been organized by Carosella, (first, second and third international congresses on HLA-G held in Paris, France), nevertheless, considerable contradictions about the source of HLA-G secretion in human placenta have persisted until now. It seems likely that technical problems, such as the lack of appropriate controls and insufficient morphological knowledge, may have led to various misinterpretations. Hunt and Geraghty, Le Bouteiller and Le Maoult in their critical letters (Hunt and Geraghty, current issue; Le Bouteiller, current issue; Le Bouteiller, current issue; Le Maoult \textit{et al}., current issue) also come to this conclusion. However, if compared, their previously published reports (Ishitani \textit{et al}., 2003; Morales \textit{et al}., 2003; Menier \textit{et al}., 2004) show major discrepancies and interestingly, the recently written critical comments by Hunt and Geraghty, Le Bouteiller and Le Maoult again reflect this discordance.

The dispute about the source of HLA-G5 secretion

Whereas the two scientific groups from Le Bouteiller and Hunt argue that the villous trophoblasts secrete soluble HLA-G, the group of Carosella mainly attributes the intron 4-containing HLA-G\textsubscript{5} or \textit{–G}6 to fetally derived erythroblasts and minimally also to extravillous cytotrophoblasts and endothelial cells. In contrast, Morales \textit{et al}.

(2003) found maternal intervillous space blood and fibrin but not fetal blood cells stained with anti-intron 4 antibodies. Ishitani \textit{et al}.

(2003) have shown villous trophoblast labelling in serial sections stained with mAb 16G1 but not with mAb W6/32. No explanation has been provided so far by these scientists as to why the well-known antibody W6/32, with the proven ability to bind HLA-G5 and therefore frequently used in sandwich ELISAs, does not stain villous trophoblasts, which would be expected in case of \textit{–G5} secretion. This also applies to mAb 4H84. In spite of the fact that this mAb binds the \(\alpha\) 1 domain regardless of the glycosylation status (McMaster \textit{et al}., 1998; Blaschitz \textit{et al}., current issue), one has to assume that HLA-G is not expressed in villous trophoblasts or in the mesenchyme or erythroblasts. At this point, it is also necessary to mention that mAb 4H84 does not require association with \(\beta\)2m to generate the conformational epitope as it has been proposed recently by Hunt (Hunt and Geraghty, current issue). But why does antibody 5A6G7 stain erythroid progenitor cells and 4H84 does not (Figure 1)? The suggestion by Menier \textit{et al}.

(2004) that different glycosylation or conformation might account for this discrepancy seems implausible since we have proved again the well-characterized mAb 4H84 (McMaster \textit{et al}., 1998) even on unglycosylated recombinant HLA-G; and if it was true that a novel isoform lacking the \(\alpha\) 1 domain but containing the intron 4-derived peptide (Menier \textit{et al}., 2004) was detected by mAb 5A6G7, investigations about secretion and function would have to start at the bottom.

Comments on criticism of our methodology

Soluble HLA-G research history has indeed taught us not to neglect technical details. It is, e.g. no longer worth considering placenta macrophages (Yang \textit{et al}., 1996) or endothelial cells (Blaschitz \textit{et al}., 1997) as sources of HLA-G secretion, since it has been clearly shown meanwhile that the antibodies 87G and BFL.1, which led to these assumptions, caused misinterpretations due to their strong affinity to various Fc (crystalline fragment) receptors (Sedlmayr \textit{et al}., 2002; Honig \textit{et al}., 2005).

We have now presented a very comprehensive study based on profound morphological understanding, paying special attention to controlling every single technique positively and negatively. A simple loss of reactivity of certain reagents, e.g. mAb 16G1, as asserted by Le Maoult \textit{et al}.

(current issue) can therefore be absolutely excluded.

Perfectly matching results obtained from analysing different HLA-G-expressing cells and tissues in combination with positive and negative controls by employing various techniques cannot simply be brushed aside by assuming methodological errors.

Immunohistochemistry

Depending on a subjective interpretation of staining patterns, immunohistochemistry is probably a more difficult matter than other methodologies. However, the scientific community has to make its own decision by looking at the panels. Unfortunately, some published pictures are of bad quality, hardly convincing and leaving some of the clearly visible stainings uninterpreted. This has been true for some of the previously shown HLA-G5 immuno-localizations where, e.g. the

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mesenchyme was stained but not commented (Chu et al., 1998; Ishitani et al., 2003).

Fully aware of problems which might occur by doing immunohistochemistry, we have been improving and controlling our protocols very carefully and are conscious of the limitations of this technology. According to Le Bouteiller’s criticism (current issue) concerning the detection limit of immunostaining, we believe that two copies of a gene naturally expressed in a cell at an extremely low level might lead to protein amounts too low to be detectable immunohistochemically. However, other serious groups analysing the in vivo situation would have experienced the same problem. In addition, this argument would not explain why a staining occurs after formalin treatment in pan HLA-G negative cells.

Obviously, Le Maoult et al. (current issue) misinterpreted results which we published previously together with Le Bouteiller (Blaschitz et al., 1997). This misinterpretation might have happened due to imprecise understanding of the morphological architecture of cell columns. The former paper showed that the highly proliferative extravillous trophoblast cells close to the mesenchymal core were the only trophoblasts which were faintly labelled with mAb BFL.1 and even less (data not shown) with mAb 16G1. At that time, we showed that these few cells were not labelled with mAb 87G, and that HLA-G expression was not initiated until the trophoblasts have migrated further from the mesenchyme. The highly proliferative extravillous trophoblasts are not the same cells which are known to express HLA-G1 or even pan HLA-G (Figure 2). Additionally, the previous paper (Blaschitz et al., 1997) described the lack of 16G1 labelling in 87G positive Jeg3 cells.

**Peptide control**

The pre-absorption of polyclonal antibodies with the corresponding highly purified antigen or synthetic peptide is a common specificity check and a helpful tool for various applications. This is not the case for monoclonal antibodies: Since all of them have the same epitope-binding groove they will of course be blocked by an admixture of antigen or peptide. This control will block both the specific reaction and also cross reactions. Cross reactions are defined as a binding to epitopes present on molecules other than the one with which the animal has been immunized or to artificially generated epitopes which might have emerged during antigen processing (Alexander and Dayal, 1997). Nevertheless, non-specific staining caused by non-epitope interactions such as FcR binding or hydrophobic interactions can be identified by pre-adsorption of monoclonal antibodies, but this kind of background is also displayed by the generally used isotype-matched negative controls.

**Cell lines**

It has been published that both cell types, villous and extravillous trophoblasts, secrete HLA-G. Therefore, it was advantageous for our study to isolate the entire first trimester trophoblast population. Since no HLA-G5 was produced by this ‘trophoblast mixture’, we saw no reason to further subdivide these cells. In addition, we decided not to cultivate the primary cultures for more than 24 h to avoid alteration of their phenotype due to culture-induced differentiation or de-differentiation processes.

Of course, our experimental design is therefore not directly comparable with those of others (Solier et al., 2002; Morales et al., 2003).

**Figure 1.** Paraffin sections of first trimester placenta showing fetal blood cells in a vessel of the chorionic plate. Antibody 5A6G7 stains erythroblasts, whereas 4H84 does not stain any of the cells.

**Figure 2.** First trimester cryosection stained for pan HLA-G with mAb 4H84—extravillous trophoblasts (EVCT) located distally to the mesenchymal core (M) increase their invasive phenotype and express HLA-G. The highly proliferative extravillous trophoblasts next to the mesenchymal core are not labelled as is the case for villous cytotrophoblasts (VCT) and syncytiotrophoblasts (ST).
However, our intention was not to repeat other studies but to search for HLA-G5 under strictly controlled conditions reflecting in vitro situations in some of our experiments.

We used the trophoblast hybrid cell line AC1-M59 (Gaus et al., 1997) as a model for HLA-G-expressing chorion leave trophoblasts but not for villous cytotrophoblast as suggested by Le Bouteiller (current issue).

**Western blot**

The recombinant HLA-G-free heavy chain was a good control to check the antibody binding abilities, and since it was used in combination with the full-length proteins from transfected cells, we disagree with Le Bouteiller that it was not a useful control. The HLA-G1 transfected over expressing cell line LCL 722.221 also left a full-length membrane-bound molecule in the culture supernatant. This observation was also made by other scientists and might be due to cell membranes which entered the media as vesicles or cell fragments having escaped the centrifugation step. Additionally, considering the overexpression in transfecants, we used, as indicated, only 5–10% of their total proteins when comparing them with trophoblasts.

**PCR experiments**

Reading the comments of Hunt and Le Bouteiller very carefully, we come to the only possible conclusion that they deduce negative PCR data from our Figure 4B (Blaschitz et al., in press) for all soluble HLA-G variants. However, this is not the case, as demonstrated unequivocally by gel photos of HLA-G5 and -G6 PCR products of first trimester trophoblast in the same figure. Additionally, relative amounts were mentioned in the paper: ‘The soluble forms revealed only low abundance of 1.5 % and 0.2 % for HLA-G5 and -G6 respectively’. This low abundance of HLA-G5 and -G6 RNA is not in contrast to previous results. To demonstrate this, we turned our attention once again to the literature of the groups mentioned: Kirszenbaum and colleagues demonstrate, even before soluble HLA-G variants were discovered, very clearly (Kirszenbaum et al., 1994) in their Figure 2 that PCR with an exon 3/3′ UTR primer set reveals visible products only for HLA-G1 and -G4 in first trimester trophoblast cells, although HLA-G5, if present in substantial amounts, should have been amplified too. Similar experiments were performed for peripheral blood mononuclear cells (PBMC) and umbilical cord blood mononuclear cells (CBMC) (Kirszenbaum et al., 1995). PBMCs again revealed only HLA-G1 and -G4, but CBMCs showed a weak band above the HLA-G1 product which was suspected to descend from HLA-G5. Whether the minute amounts that were detected in this study have physiological relevance must be discussed elsewhere. In a study of Morau and colleagues (Moreau et al., 1995), HLA-G5 signals are hardly recognizable in two of three first trimester samples a small number of HLA-G5 fragments were observed. Generally, the expression of the soluble HLA-G mRNA isoforms is much lower than that of the membrane-bound isoforms’ (Hviid et al. 2003).

Other studies, some of which were mentioned to conflict with our data do analyse either membrane-bound (Ishitani and Geraghty, 1992; Onno et al., 1994) or soluble variants (Fujii et al., 1994; Chu et al., 1998; Solier et al., 2002; Morales et al., 2003) only and therefore should not be compared to our data. In conclusion, we were not able to detect conflicting results regarding the expression level of HLA-G splicing variants.

The remark that we should have used primers that were used previously for this kind of analysis is difficult to follow, since variant-specific primer pairs have, to our knowledge, not been described so far in literature. Apart from the primers G.2–5 (Kirszenbaum et al., 1995), G.3–5 (Kirszenbaum et al., 1994) and intron 4-specific primers, which lead to a partial splicing variant-specific reaction, previously used oligonucleotides were not isotype specific at all. Since quantification by RT–PCR requires amplification of a single definite product, such primers are not feasible. Nevertheless, our primers are very close to the primers described previously: G2-3 for and G2-4 for resemble G2-5, but the part of exon 5 was replaced by exon 3 and 4 sequences, respectively. HLA-G 2ndS-Primer binds to almost the same position as GA5.2 (Onno et al., 1994) (3′ end identical) and G.257 (Kirszenbaum et al., 1994) (3′ end 2 bases upstream) and confirms therefore the already established HLA-G specificity. Nevertheless, we included HLA-C which is known to be expressed in trophoblast, as a specific control in our analysis. Regarding the suspected HLA-J amplification, an alignment of HLA-G 2ndS-Primer with the respective positions of HLA-J and -C demonstrates lots of mismatches, making co-amplification implausible (Figure 3). The same holds true for the variant-specific primers with at least three mismatches if aligned to HLA-J. Together with the clear-cut bands in the gel and the fact that sequencing of the three first trimester trophoblast products—as well as of many others not mentioned in the article—never revealed a non-HLA-G sequence, we do not know why specificity is suspected to be a problem.

Hunt complains about our preamplification protocol because of primer competition, but all preamplifications covering more than one isotype are the same regarding this issue. For our method, this is more a feature than a drawback, since the housekeeper may be co-amplified for normalization without primer bias. However, we were initially interested in using G45-65 and G1225 (Morales et al., 2003) for preamplification, but this failed to provide satisfactory success. Moreover, PCR conditions were not mentioned by Morales and colleagues.

In order to avoid problems with biased PCR, e.g. due to different amplification efficiencies and product length (CyberGreen fluorescence is directly size dependent), a dilution series of quantified and purified HLA-G1, -G5, -G6 products were used for standardization. Using the same master mix, these standard reactions were conducted in parallel with the samples. Due to the fast rotation of the reaction tubes in the Rotorgene device, all samples have virtually identical PCR conditions. Hep-G2 cells have been included as a negative control, and all reactions were negative. Since this information is neither necessary nor supportive, this result was not mentioned in the publication. The same holds true for the positive control Jeg3 which revealed about half the amounts of villous chorion regarding HLA-G1 and -G5 transcripts. The RNA concentration of different samples was adjusted prior to the preamplification by β-actin-specific PCR. For fine adjustment, β-actin was included in preamplification and also standardized with the respective dilution series-derived calibration curves. However, the main message of these RT–PCR experiments is the relation of HLA-G1 to –G5 and –G6, and this is completely independent of minor differences regarding the mRNA input.

AGAGGAGACACGGAAAAH HLA-G
CCTACAGAAGCTGCGCCCH HLA-J
CCGGAAGACACAGAACTAA Cw*1510

Figure 3. Alignment of different HLA-sequences with the HLA-G-specific part of HLA-G2ndS-Primer.
ELISA

We did not generate a highly purified standard for absolute quantifications in our ELISA experiments, because the main purpose for this study was to show the relative sensitivities of 16G1-based approaches compared with assays based on other HLA-G antibodies capable of detecting HLA-G5 and -G6 plus shed or cleaved HLA-G1 and all α1 domain-containing isoforms.

Our ELISA findings are in line with Solier’s observations (Solier et al., 2002) who additionally found an interesting inversion in sensitivity when analysing supernatants of villous cyto- and syncytiotrophoblasts in comparison to transfected cells indicating a higher secretion by villous trophoblasts than by HLA-G5 transfecants. This is all the more surprising, since transfected cells over-express HLA-G5, and although Le Bouteiller called for awareness of over-expressing transfected control cells, this result was not discussed. We also observed such an inversion when analysing aqueous tissue extracts of term placenta (Figure 4A): At first glance, our 16G1-based data might suggest, that there is HLA-G5 in villous tissue; however, there is no explanation why the other ELISA systems which were much more sensitive, even for HLA-G5 (721.221 HLA-G5 supernatant in Figure 4A), failed to detect this signal. We hypothesize that there are considerable false positive ELISA signals when using 16G1 as coating antibody in combination with biotinylated anti-classical HLA class I antibodies such as W6/32. Figure 4B compares the 16G1-W6/32-based detection of HLA-G5 and the ‘nonsense format’ 16G1-TP25/99. TP25/99 is known to recognize all HLA class I molecules except HLA-G (Rebmann et al., 1999). This finding, already mentioned in our recent article, suggests that a cross-reaction of 16G1 to some classical HLA class I molecules cannot be excluded (Figure 4B). This possibility was further supported in 16G1 Western blots showing a strong 42kD band at the classical HLA class I molecular weight size (Blaschitz et al., 2005, this issue).

Conclusions

Besides all technical details, intron 4-containing HLA-G protein could not be convincingly detected in placental tissues by employing well-established, antibody-based standard techniques such as ELISA, Western blot and immunohistochemistry. This, of course, does not exclude the possibility that some novel, extremely sophisticated and sensitive methods might be able to display intron 4-containing HLA-G protein in vitro or in vivo in the future, but the weak evidence for its existence provided so far has not been based on such superior technologies.

In the light of increasing investigations of soluble HLA-G in a clinical context, we strongly agree with Sargent (2005, this issue) that it is of tremendous importance to know whether soluble HLA-G is mainly generated by alternative splicing or rather by shedding or cleavage.

The common usage of the term ‘actively secreted isoforms’ for HLA-G5 and -G6 suggests that soluble HLA-G was deliberately produced by certain cells, in a controlled way, to serve for a special purpose.

Shedding and cleavage are normal processes in the decay of membrane-bound proteins. If soluble HLA-G were to consist mainly of these decay products it could, of course, still be of diagnostic relevance, e.g. in the case of cell phenotype alteration and changes in cell metabolism or cell cycle under pathologic conditions. However, it is still questionable whether these kinds of soluble molecule fragments could occur in considerable amounts in human body fluids.

Figure 4. (A) HLA-G5 transfectant supernatant (CS) and aqueous extract from term placenta villous trees analysed with three different sandwich-enzyme-linked immunosorbent assay (ELISA) approaches using biotinylated (Biot.) detection antibodies and a streptavidin-based signal amplification system: Bars reflect mean optical density (OD)-values (+SEM) of three different term placentas, each analysed in duplicate. Isotype control (IC) values were subtracted. (B) Aqueous term placenta extract and 1/3 diluted HLA-G5 transfectant supernatant analysed with an HLA-G5 ‘specific’ assay in comparison with a ‘nonsense-format’ detecting classical HLA-1 captured by 16G1. Bars reflect mean OD-values (+SD). Samples were tested in duplicates. Isotype control values were subtracted.

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Our investigation revealed several lines of evidence, that the main mechanisms for the generation of soluble HLA-G are indeed shedding and cleavage of HLA-G1, at least for soluble HLA-G derived from the human placenta.

**Perspectives**

Scientific progress in the field of soluble HLA-G research would benefit from enhanced international co-operations. In this context, it is worth mentioning that a European network of excellence the EMBryo Implantation Control (EMBIC) has been established. EMBIC-work packages focusing on the role of HLA-G in human implantation appear to be a promising approach to overcome recent misunderstandings by intensive scientific exchange between different laboratories all over Europe. Additionally, the quality of HLA-G research would improve significantly by introducing double-blinded antibody evaluations according to the guidelines of the cluster of differentiation (CD) commission.

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