Cutting Edge: Soluble HLA-G1 Triggers CD95/CD95 Ligand-Mediated Apoptosis in Activated CD8⁺ Cells by Interacting with CD8

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Cutting Edge: Soluble HLA-G1 Triggers CD95/CD95 Ligand-Mediated Apoptosis in Activated CD8^+ Cells by Interacting with CD8^1

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The nonpolymorphic soluble HLA-G1 (sHLA-G1) isoform has been reported to be secreted by trophoblast cells at the materno-fetal interface, suggesting that it may act as immunomodulator during pregnancy. In this paper, we report that affinity-purified β2-microglobulin-associated sHLA-G1 triggered apoptosis in activated, but not resting CD8^+ peripheral blood cells. We demonstrate by Western blotting that sHLA-G1 enhanced CD95 ligand expression in activated CD8^+ cells. Cytotoxicity was inhibited by preincubation of the cells with a CD95 antagonist mAb (ZB4) or a soluble recombinant CD95-Fc, indicating that apoptosis is mediated through the CD95/CD95 ligand pathway. Finally, we show that such sHLA-G1-induced apoptosis depends on the interaction with CD8 molecules, with cell death being blocked by various CD8 mAbs. The Journal of Immunology, 2000, 164: 6100–6104.

During pregnancy, the fetus can be considered as a semi-allogenic allograft. However, the fetus is tolerated by the mother, suggesting an induction of tolerance against paternally derived Ags. The mechanisms that account for such a tolerance are still not clearly understood. The fetus is never in direct contact with maternal tissues; therefore, many of the tolerance mechanisms may occur only at the materno-fetal interface in the placenta. One of these interfaces is the site of implantation where fetally derived extravillous cytotrophoblast cells invade the decidua basalis and encounter maternal immune cells such as NK cells, T cells, and macrophages (1). The lack of expression of HLA-A and -B class I molecules on these trophoblast cells and, in contrast, the expression of HLA-G class Ib may contribute to the proper outcome of pregnancy (2). One of the major structural characteristics of the HLA-G gene is its potentiality to be transcribed in alternatively spliced isoforms, including membrane-bound and soluble proteins (3, 4). The HLA-G1 soluble form (sHLA-G1) is encoded by a spliced transcript containing part of intron 4, which generates a stop codon that results in the deletion of the transmembrane and cytoplasmic domains, yielding a 37-kDa soluble protein that associates to β2-microglobulin (β2-m) (4). Such intron-retaining soluble isoform appears to be a unique structural feature of HLA-G that is not observed with other soluble HLA class I molecules. sHLA-G1 was shown to be secreted during pregnancy (5–7). Some placental cells, including extravillous cytotrophoblast, macrophages in chorionic villi, and endothelial cells lining fetal placental blood vessels, have been identified as sHLA-G secretors (8–11).

In this report, we show that purified sHLA-G1 triggers in vitro apoptosis of activated CD8^+ cells by interacting with the CD8 molecules. We found that sHLA-G1 enhanced CD95 ligand (CD95-L) expression in activated CD8^+ cells and that apoptosis was the consequence of a CD95/CD95-L interaction.

Materials and Methods

Cell lines and mAbs

Jurkat cell line was provided by Dr. B. Rubin (Centre National de la Recherche Scientifique, Toulouse, France). PG CD8 is a HLA-B^*2705-restricted CD8^+ synovial cytotoxic T cell line (12). These cells were cultured in IL-2 (150 IU/ml) and activated by PHA (1 μg/ml) every 2 wk and cultured in IL-2 (150 IU/ml) for the rest of the time. The following mAbs were used: 7C11 and ZB4 (Immunotech, Marseille, France); CD95 agonist and antagonist, respectively; BL4 CD4 (gift of Prof. J. P. Revillard, Institut National de la Santé et de la Recherche Médicale U503, Lyon, France); W6/32, HLA class I heavy chains associated with β2-m; IP48 and CD8^8; CD8α-chain, produced locally; and CD95-L (clone 33; Transduction Laboratories, Lexington, KY). Soluble recombinant CD95-Fc was obtained by fusion of the extracellular domain of CD95 to the Fc fragment of human IgG1, transfection in Chinese hamster ovary cells, and recovery from the cell culture supernatant.

Purification of sHLA-G1 and sHLA-B7 proteins

β2-m-associated sHLA-G1 and sHLA-B7 were purified from culture supernatants of JAR cell line transfected with sHLA-G1 encoding cDNA (13) or

Abbreviations used in this paper: s, soluble; β2-m, β2-microglobulin; CD95-L, CD95 ligand.
of C1R cell line transfected with cDNA encoding sHLA-B7 (14), respectively, using immunoaffinity column, as has been described (13). Briefly, N-hydroxysuccinimide-activated Sepharose 4 fast flow (Pharmacia Biotech, Uppsala, Sweden) was incubated with W6/32 HLA class I mAb (5 mg protein/ml Sepharose) overnight at room temperature. Before use, the column was blocked with 100 mM ethanolamine (pH 9.0) overnight. After washes with PBS, 100–200 ml culture supernatants were applied onto the column overnight at 4°C. After washes with PBS, bound Ag was eluted with 0.1 M glycine buffer (pH 11.5) and neutralized with 1 M Tris buffer (pH 7.5). Ag purity was confirmed by SDS-PAGE and Western blotting, as previously described (15).

CD8+ cell preparation and measurement of apoptosis
PBMC from healthy donors were isolated by centrifugation of heparinized blood on a layer of Ficoll-Hypaque (Lymphoprep; Pharmacia Biotech). Cells were washed and resuspended in RPMI 1640 with Glutamax (Life Technologies, Cergy-Pontoise, France), 10% FCS, sodium pyruvate (1 mM), and antibiotics. Cultures were maintained for 3 days in the presence or not of PHA (5 mg/ml, Sigma, St. Louis, MO). After PHA activation, viable cells were depleted of CD4+ cells by adherence onto plastic flasks coated with CD4 mAb (BL4). Such PBMC suspensions contained an average of 67% (±15%) of CD8+ cells and less than 5% of CD4+ cells as measured by flow cytometry CD8-FITC and CD4-PE mAb binding, respectively. Viable CD8+ cells (10^6/ml) were incubated in 96-well microplates with sHLA molecules or mAbs at indicated concentrations. Cell death was then evaluated by fluorescence microscopy measurement of nuclear condensation and fragmentation after Hoechst 33342 staining (Sigma) (15). Results were expressed as percentage of specific apoptosis, according to the following formula: % specific apoptosis = [(% of apoptotic treated cells – % of apoptotic control cells) x 100]/(100 – % of apoptotic control cells). Apoptosis was also evaluated by the detection of phosphatidylinerse expression by flow cytometry after addition of FITC-labeled human Annexin V (Bender MedSystems, Vienna, Austria) (16).

CD95-L-induced cytotoxicity assay
The CD95-L-dependent cytotoxicity was measured by [3H]DNA released from Jurkat cells induced by CD95-L-producing cells, as previously described (17, 18). Jurkat cells were pulsed for 12 h with 20 μCi/ml of [3H]Th (Amersham, les Ulis, France). After washes, [3H]-labeled Jurkat cells (0.2 × 10^6 cells/ml) were incubated with resting or PHA-activated CD8+ cells previously treated for 15 h with sHLA-G1 (1 μg/ml) or sHLA-B7 (1 μg/ml) at a ratio of one Jurkat cell for three CD8+ cells, with or without the antagonist CD95 mAb ZB4 (2 μg/ml). After 12 h of culture, [3H]DNA release induced by apoptosis of Jurkat cells was measured using a Packard direct beta counter (Packard, Meriden, CT). Results were expressed as percentage of cytotoxicity, according to the following formula: % cytotoxicity = [(cpm spontaneous – cpm sample) x 100]/cpm spontaneous. Cytotoxicity inhibited by ZB4 was considered as specific to CD95-L-dependent apoptosis.

Immunofluorescence assays
Cells were stained with CD8-FITC or CD4-PE (Immunotech) or CD25-FITC (PharmMingen, San Diego, CA) mAbs for 30 min at 4°C, washed, and analyzed on a Coulter (Margency, France) Epics Elite flow cytometer gated to exclude nonviable cells.

Western blotting
Western blotting was performed as described (13). Briefly, cell lysates were separated on a 12% SDS-PAGE and transferred on nitrocellulose membranes. Membranes were blocked with PBS/0.1% Tween 20/5% non-fat dry milk and incubated with CD95-L mAb and then with peroxidase-labeled rabbit anti-mouse IgG and enhanced chemiluminescence Western blotting detection reagent (Amersham).

Results and Discussion
sHLA-G1 induces apoptosis of activated CD8+ cells in a dose- and time-dependent manner
To investigate the effect of sHLA-G1 on CD8+ cells, PBMC were activated for 3 days by PHA, depleted of CD4+ cells, and incubated with sHLA-G1, sHLA-B7, or an agonist CD95 mAb (7C11), used as positive control. We first analyzed induction of apoptosis by Hoechst staining. sHLA-G1 reproducibly induced specific apoptosis of PHA-activated CD8+ cells (Fig. 1A). By comparison, sHLA-B7 induced only a slight or no apoptosis, whereas CD95 mAb 7C11 triggered a stronger specific killing. Twenty eight ± 10% of CD8+ cells were estimated to be activated by PHA, as measured by expression of the IL-2R α-chain CD25 (data not shown). This suggested that about 50% of activated CD8+ cells were killed by sHLA-G1, compared with 100% of killing by the CD95 mAb. Almost no apoptotic effect of sHLA-G1 or sHLA-B7 was detected by this technique on resting CD8+ cells (Fig. 1A). Apoptosis was then evaluated by Annexin V staining (Fig. 1B). When added to PHA-activated CD8+ cells, sHLA-G1, like CD95 mAb, triggered externalization of phosphatidylserine measured by the binding of Annexin V as a typical feature of apoptosis (11.8 and 17.7% specific apoptosis, respectively), whereas both sHLA-G1 and CD95 mAb had little effect on resting CD8+ cells (5.5 and 8.1% of specific apoptosis, respectively). As control, sHLA-B7 had no specific effect on resting and activated CD8+ cells (no specific apoptosis).

sHLA-G1 induced specific apoptosis of PHA-activated CD8+ cells at a concentration as low as 0.25 μg/ml with a maximum effect at 1 μg/ml (Fig. 2A). Maximum sHLA-G1-induced apoptosis was observed after 24 h of incubation with a kinetics comparable to the CD95 mAb-induced apoptosis (Fig. 2B).

sHLA-G1-induced apoptosis of activated CD8+ cells is mediated by CD95/CD95-L interaction
Knowing that activated T cells express CD95 receptors (19) and are sensitive to CD95-L-mediated apoptosis (20), we examined the contribution of CD95/CD95-L interaction in sHLA-G1-induced apoptosis. We first investigated by Western blotting whether sHLA-G1 increased expression of CD95-L in PHA-activated CD8+ cells (Fig. 3A). Using a CD95-L-specific mAb, one specific band in the range of 37 kDa was clearly detectable in PHA-activated CD8+ cells incubated with sHLA-G1 for 24 h (lanes 1 and 2) or 12 h (lanes 3 and 4). Depending on cell concentration, no signal or a faint band was revealed in control untreated PHA-activated CD8+ cells (lanes 5 and 6, respectively), whereas no signal was observed in resting CD8+ cells (lanes 7 and 8). These results demonstrated that sHLA-G1 enhanced CD95-L expression in PHA-activated CD8+ cells.

To investigate the functionality of such a CD95-L expression in PHA-activated CD8+ cells treated with sHLA-G1, we then used the CD95+ Jurkat cells, which are susceptible to CD95-L-mediated apoptosis (17, 18). PHA-activated CD8+ cells were incubated either with sHLA-G1 or sHLA-B7 for 15 h and then cocultured for a further 12 h with [3H]thymidine-labeled Jurkat target cells in the presence or absence of the antagonist CD95 mAb ZB4. PHA-activated CD8+ cells treated with sHLA-G1 induced [3H]thymidine release from Jurkat cell (Fig. 3B). Preincubation with the antagonist CD95 mAb ZB4 completely abolished this effect, suggesting that sHLA-G1 induced expression of functional CD95-L. In contrast, sHLA-B7 did not induce specific [3H]thymidine release (Fig. 3B). Consistently with the previous data, we next demonstrated that a preincubation with the same antagonist CD95 mAb ZB4 prevented not only 7C11- but also sHLA-G1s-induced apoptosis of PHA-activated CD8+ T cells (Fig. 3C), whereas no lysis was observed in nonactivated cells (data not shown). Similar results were obtained when CD95-Fc, which blocked interaction between CD95 and CD95-L, was used instead of ZB4. These results further
confirmed that sHLA-G1-induced apoptosis is mediated by the CD95/CD95-L pathway.

**sHLA-G1-induced apoptosis is dependent on interaction with CD8 molecule**

sHLA-G1 may interact with CD8<sup>+</sup> T cells through either HLA-G-restricted TCR (21) or the CD8 molecules (22, 23). We investigated the contribution of CD8. PHA-activated CD8<sup>+</sup> cells were preincubated for 1 h with CD8 mAbs before the addition of sHLA-G1, sHLA-B7, or CD95 mAb. Apoptosis was determined by fluorescence microscopy measurement of nuclear condensation and fragmentation after Hoechst 33342 staining. Results are expressed as percentage of specific apoptosis, as described in Materials and Methods. Spontaneous apoptosis in medium alone did not exceed 15%. Values are the mean ± SD of ten experiments. B, Flow cytometry analysis after Annexin V-FITC staining. PHA-activated CD8<sup>+</sup> cells or control resting CD8<sup>+</sup> cells (10<sup>6</sup> cells/ml) were incubated with culture medium or with sHLA-G1s (1 μg/ml), sHLA-B7 (1 μg/ml), or the CD95 agonist mAb 7C11 (1 μg/ml). After 24 h, cells were stained with Annexin V-FITC and analyzed by flow cytometry. Percentages of Annexin-V-positive cells are indicated in each panel. Spontaneous apoptosis in medium alone did not exceed 20%. These results are representative of six independent experiments.

![FIGURE 1.](image)

**FIGURE 1.** sHLA-G1 induces apoptosis of activated CD8<sup>+</sup> cells. A, PBMC were activated for 3 days with PHA (5 μg/ml). After removal of dead cells and depletion of CD4<sup>+</sup> cells, PHA-activated CD8<sup>+</sup> cells (10<sup>6</sup> cells/ml) were incubated for 20 h with sHLA-G1s (1 μg/ml), sHLA-B7 (1 μg/ml), or CD95 agonist mAb 7C11 (1 μg/ml). Control resting CD8<sup>+</sup> cells were similarly incubated with sHLA-G1, sHLA-B7, or CD95 mAb. Apoptosis was determined by fluorescence microscopy measurement of nuclear condensation and fragmentation after Hoechst 33342 staining. Results are expressed as percentage of specific apoptosis, as described in Materials and Methods. Spontaneous apoptosis in medium alone did not exceed 15%. Values are the mean ± SD of ten experiments. B, Flow cytometry analysis after Annexin V-FITC staining. PHA-activated CD8<sup>+</sup> cells or control resting CD8<sup>+</sup> cells (10<sup>6</sup> cells/ml) were incubated with culture medium or with sHLA-G1s (1 μg/ml), sHLA-B7 (1 μg/ml), or the CD95 agonist mAb 7C11 (1 μg/ml). After 24 h, cells were stained with Annexin V-FITC and analyzed by flow cytometry. Percentages of Annexin-V-positive cells are indicated in each panel. Spontaneous apoptosis in medium alone did not exceed 20%. These results are representative of six independent experiments.

![FIGURE 2.](image)

**FIGURE 2.** Dose response and kinetics of sHLA-G1-induced apoptosis. A, PHA-activated CD8<sup>+</sup> cells were incubated for 20 h with sHLA-G1s at indicated concentrations. Apoptosis was determined by fluorescence microscopy after Hoechst 33342 staining, and results are expressed as specific apoptosis as in Fig. 1A. Values are the mean ± SD of five separate experiments. B, PHA-activated CD8<sup>+</sup> cells were incubated either with sHLA-G1s (1 μg/ml; ○) or with the 7C11 agonist CD95 mAb (1 μg/ml; □). At indicated times, apoptosis was similarly determined. Values are expressed as the mean ± SD of three experiments.
of activation (i.e., PHA). In addition, they exclude the possibility that sHLA-G1 did interact with the TCR to induce such apoptosis. It was recently shown that CD4 cross-linking by HIV gp 120 triggered CD4+ T cell apoptosis (24). Our data confirm that apoptosis could be induced through accessory molecules.

Although unlikely, our results cannot completely exclude that CD4+ T cells were also susceptible to sHLA-G1-mediated apoptosis. Because an interaction between sHLA-G1 and CD4 is unusual for a class I molecule, a binding to other unknown receptors present on CD4+ cells therefore would be implied. It has been demonstrated that HLA-G binds to several killer cell Ig-like receptors, including Ig-like transcripts 2 and 4, expressed in particular on myelomonocytic cells (reviewed in Ref. 1). Therefore, we cannot rule out that sHLA-G1 has bound to such receptors expressed on some of these cells present in our cultures, modulating one or several of their Ag-presenting functions and thereby having indirect effects on CD8+ cells.

sHLA class Ia molecules have been reported to induce apoptosis in alloreactive CTL (25). The mechanisms underlying this phenomenon appear to be distinct from what we observed with sHLA-G1, because sHLA class Ia triggered cell death by interaction with TCR, whereas sHLA-G1 induced apoptosis by interaction with CD8. Interaction of HLA-G with CD8 was already reported to involve a conserved negatively charged loop localized in the α3 domain of the HLA-G heavy chain (22). Two main structural differences between sHLA-G1 and sHLA-B7 could explain the differential effect of these two molecules on apoptosis of CD8+. First, at position 228 within α3 binding domain to CD8, HLA-G has a valine instead of the conserved threonine residue found in all other HLA class Ia (26). Second, sHLA-G1 has a translated part of intron 4 (4), which could modify the general conformation of the CD8 binding sites. Based on these structural differences, one can predict that the CD8 binding affinity would differ between sHLA-G1 and sHLA-B7. Experiments using HLA-G/HLA-B7 chimera molecules remain to be done to investigate these possibilities. A very recent report mentioned that sHLA class I molecules, isolated from human sera, induced apoptosis in activated CD8+ but not in CD4+ T cells (27). However, these results did not discriminate between classical and nonclassical soluble HLA class I products. Therefore, it cannot be excluded that part of these soluble MHC class I molecules were HLA-G, possibly secreted by activated macrophages (10).

Because sHLA-G1 is mainly expressed in the placenta (1), we hypothesize that sHLA-G1 may, among other functions (1, 2), contribute to the elimination of CD8+ alloreactive maternal immune T cells in vivo at the materno-fetal interface through the CD95/
CD95-L pathway. Several observations favor such a hypothesis. First, sHLA-G was shown to be secreted by invading extravillous cytrophoblasts present in the decidua (10, 11). Second, a level of sHLA-G1 significantly higher than that in early abortion recently has been reported in early intact pregnancy (28). Third, few CD8 T cells have been found in the human decidua by immunohistochemistry or flow cytometry analysis (29), whereas apoptotic nuclei mainly arising from CD45 positive leukocytes recently have been detected at the same materno-fetal interface (30). These latter two observations suggest that CD8 was also present on the surface of some decidual CD561 NK cells. PHA-activated CD8 mAbs, or with IgG control (10 μg/ml), were added, and after 20 h, the percentage of apoptotic cells was determined by fluorescence microscopy after Hoechst 33342 staining. Results are expressed as specific apoptosis as in Fig. 1A. Spontaneous apoptosis did not exceed 15% with any of the agents tested. Values are the mean ± SD in three independent experiments.

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References


FIGURE 4. CD8 mAbs inhibit sHLA-G1-induced apoptosis of activated CD8+ cells. PHA-activated CD8+ cells were incubated for 1 h with medium alone, with CD8+×8 (ascite 1/1000) or IP48 (ascite 1/200) CD8 mAbs, or with IgG control (10 μg/ml). Then, sHLA-G1s (1 μg/ml), sHLA-B7 (1 μg/ml), or the agonist CD95 mAb 7C11 (1 μg/ml) was added, and after 20 h, the percentage of apoptotic cells was determined by fluorescence microscopy after Hoechst 33342 staining. Results are expressed as specific apoptosis as in Fig. 1A. Spontaneous apoptosis did not exceed 15% with any of the agents tested. Values are the mean ± SD in three independent experiments.