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Involvement of gD/HVEM interaction in NF-kB-dependent inhibition of apoptosis by HSV-1 gD

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ABSTRACT

In the present paper, we aimed to verify whether the interaction of the glycoprotein D (gD) of herpes simplex 1 (HSV-1) with the HSV-1 receptor HVEM is involved in NF-κB-dependent protection against apoptosis by gD. To this purpose, first we utilized MAbs that interfere with gD/HVEM interaction and U937 cells that naturally express human HVEM on their surface. Pre-incubation with these MAbs, but not with a control antibody, partially reverted the protection of infectious HSV-1 towards anti-Fas induced apoptosis in U937 cells. Similarly, pre-incubation of UV-inactivated HSV-1 (UV-HSV-1) or recombinant gD with the same MAbs, significantly reduced the inhibition of Fas-mediated apoptosis by UV-HSV-1 or gD, respectively, in U937 cells. Moreover, coculture with stable transfectants expressing at surface level wild type gD protected U937 cells against Fas-induced apoptosis, while coculture with transfectants expressing a mutated form of gD, incapable to bind HVEM, did not protect. Finally, UV-HSV-1 protected against staurosporine-induced apoptosis in U937 cells as well as in the CHO transfectants expressing human HVEM on their surface, but not in the control CHO transfectants, which did not express HVEM. These results suggest that signaling triggered by binding of gD to HVEM could represent an additional mechanism of evasion from premature apoptotic death exerted by HSV-1-gD in HVEM-expressing cells, disclosing new opportunities of cell death manipulation by using gD preparations.

1. Introduction

Replication and spread of viruses can be efficiently limited by a cellular apoptotic response, that represents an important, first line form of antiviral innate defense (reviewed in [1–3]).[1] Nevertheless, different viruses have evolved multiple survival strategies to escape elimination by apoptosis and interference with signals involved in the death of infected cells is one of the

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more powerful mechanisms for viruses to control non-specific resistance of the host. In fact, regulation of apoptotic pathways is exploited by a wide variety of viruses, either to permit the maintenance of viral infection or to maintain the viability of the host cell, thus enhancing the efficiency of viral replication (reviewed in [4–6]). Particularly, herpes simplex virus 1 (HSV-1) seems able to exert a fine control on apoptosis of infected cells, mainly consisting in prevention of premature cell death to guarantee that a prolonged virus replication could occur (reviewed in [7,8]). In fact, a number of HSV-1 gene products have been proved to play a role in preventing apoptosis. These include the γ134.5 protein, the infected cell protein no. 4 (ICP4), the infected cell protein no. 27 (ICP27), the Us3 protein kinase and, as recently shown, the Us11 protein [9–14]. Moreover, also the envelope glycoprotein D (gD) of HSV-1 has been shown to exert an anti-apoptotic effect [15–17]. Regarding to the latter, we have previously demonstrated that gD of HSV-1, as well as non-replicating UV-inactivated HSV-1, was able to protect U937 cells against Fas-induced apoptosis and that this effect was dependent on activation of NF-kB [17]. However, molecular events involved in cellular signalling upstream NF-kB activation have not been identified.

Binding of the envelope gD with surface cell receptors plays a central role for HSV-1 entry into the cell and for initiating the replication cycle of the virus [18,19]. Receptors for HSV-1-gD include a modified form of heparan sulfate, nectin-1 and nectin-2 and the herpes virus entry mediator (HVEM), a member of the tumor necrosis factor receptor family [19].

We have recently observed that the interaction of gD with HVEM can be involved in NF-kB activation by non-replicating HSV-1 [20]. Thus, we asked whether the same interaction is involved in protection against apoptosis exerted by HSV-1 or its gD in HVEM-expressing cells. To answer this question we tested for a possible role of HVEM in gD-dependent anti-apoptotic activity of HSV-1. Here, we show that, in HVEM-expressing U937 cells, prevention of gD/HVEM interaction by appropriate monoclonal antibodies or by usage of transfectants expressing a mutated form of gD, incapable to bind HVEM, significantly reduced protection against Fas-mediated apoptosis by HSV-1/gD. Moreover, human HVEM expression by stable transfection rendered CHO cells sensitive to HSV-1-induced protection against staurosporine induced apoptosis, whilst control CHO transfectants, which did not express human HVEM, were resistant to such a protective action. Therefore, our results support a role for HVEM in the anti-apoptotic activity of HSV-1/gD.

2. Materials and methods

2.1. Virus and cells

A "F" strain of HSV-1, originally obtained from ATCC, was used in all experiments. Virus stocks were produced, titered in Vero cells and stored in aliquots at −80 °C. To obtain UV-inactivated virus (UV-HSV-1), the HSV-1 suspension was placed in Petri dishes and exposed for 210 s to UV light at an intensity of 30 W using a germicidal lamp situated 10 cm above the sample. Lack of infectivity was tested by titering aliquots of UV-HSV-1 in Vero cells. For experiments of protection against apoptosis by infectious or UV-inactivated virus, approximately 6×10^4 U937 cells in 96-well plates were exposed to virus inoculum for 1 h, at a multiplicity of infection (MOI) of 50 PFU/cell for infectious HSV-1 and, usually, at a MOI of 200 PFU/cell for UV-HSV-1, respectively, 24 h after cultures had been split. The MOI used were chosen on the basis of our previous study [17] and preliminary experiments showing that any further increase in the MOI of UV-HSV-1 failed to improve protection from apoptosis. Subsequently, virus inoculum was replaced by fresh growth medium and cells were subjected to successive experimental procedures.

Vero (originally obtained from the American Type Culture Collection, ATCC) and I143tk$^-$ cells (kindly provided by Prof. Gabriella Campadelli-Fiume, University of Bologna, Bologna) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% FCS (Sigma–Aldrich, St. Louis, MI). CHO cells (originally obtained from the European Collection of Cell Cultures, ECACC) were propagated in HAM'S medium supplemented with 10% of FCS (Sigma–Aldrich). U937 cells (originally obtained from the Istituto Zooprofilattico, Brescia, Italy), were propagated in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% FCS (Sigma–Aldrich). Cell lines were cultured at 37 °C in a 5% CO_2 incubator. Cell viability was assessed by a standard trypan blue exclusion test.

2.2. Antibodies and reagents

Anti-gD monoclonal antibody (MAb) 1D3 and purified recombinant gD [gD-1(306t)], were kindly provided by Dr. Gary H. Cohen and Dr. Roselyn J. Eisenberg, University of Pennsylvania, Philadelphia, PA. Anti-gD 1103/H170 MAb, anti-gD HD1 MAb and anti-gC 1104/R633 MAb, were a gift from Prof. Bernard Roizman, University of Chicago, Chicago, IL. MAbs 1D3 and HD1 neutralize HSV infection of cultured cells and blocks HSV-1 binding to HVEM. Anti-HVEM ANC3B7 (SC-65284) MAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was purchased from ICN Biomedicals (Aurora, OH). Anti-human Fas antibody, clone CH11, was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse IgG1 isotype control MAb was purchased from R&D System (Minneapolis, MN). Staurosporine was purchased from Sigma–Aldrich.

2.3. Generation of HVEM expression-construct

An HVEM expression-construct was generated using cDNA obtained from HVEM expressing U937 cells. To this purpose, total RNA was extracted from U937 cells using the "TRIZOL RNA extraction kit" (Gibco). An amount of 1.5 μg of total-RNA was then reverse-transcribed to cDNA using 60 units of AMV reverse transcriptase. The reverse transcription was primed with a mixture of oligo(dT)15 and random hexamer primers and performed in presence of a pool of nucleotides consisting of 1 mM dGTP, dATP, dTTP and dCTP (Promega, Minneapolis, MN). Twenty units of SUPERase-IN (Ambion, Austin, TX) were added to each reaction mixture. The mixture, containing the RNA template and the primers, was first heated at 70 °C for 10 min, chilled on ice, and after the addition of the other components, incubated at 42 °C for 45 min, shifted at 52 °C for 45 min, and

then heat-inactivated at 95 °C for 5 min. To generate a full-length HVEM expression construct, the obtained cDNA was then amplified by PCR under the following conditions: 1 min at 95 °C, 45 s at 60 °C and 1 min at 72 °C. A specific primer pair designed on the basis of the ''Homo sapiens tumor necrosis factor receptor superfamily, member 14'' cDNA sequence (NCBI GenBank, accession no. NM_003820.2) was used. The forward primer contained an EcoRI site upstream of the start codon: 5′-CGGAATTTCATGGAGCCTCCTGGAGACTGG-3′, and the reverse primer contained NotI site downstream the stop codon: 5′-ATTGCGGCCGCTCAGTGGTTTGGGCTCCTC-3′. The amplification product was then double digested with EcoRI/NotI enzymes, cloned into the commercial expression vector pcDNA3.1 (Invitrogen, Carlsband, CA). The generated expression-construct, designated as pcDNA-HVEM, was amplified and sequenced to verify the exact sequence (MWG Biotech, Ebersberg, Germany).

2.4. Generation of stable transfectants

The pcDNA-HVEM and pcDNA3.1 plasmids were transfected into CHO cells. For transfection, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FCS at 37 °C using standard procedures. One day before transfection, cells were split and distributed in new culture flasks. Transfections were performed in 6-well plates, at 5×10^5 cells/well in 2 ml DMEM with 10% FCS. For each transfection, 3 μg plasmid DNA and 10 μl Lipofectamine™ 2000 (Invitrogen) were used. The pcDNA-HVEM and pcDNA3.1 plasmid DNA was pre-incubated with Lipofectamine™ 2000 for 30 min at room temperature in 200 μl DMEM without serum before addition to the cells and incubation at 37 °C for 4 h. Transfected cells were then washed and, after the addition of 2 ml complete medium, were incubated for 3 days at 37 °C. After this incubation time, transfected cells were further grown under G418 (Invitrogen) selection. Subsequently, cells were trypsinized, distributed in 96-well plates by single-cell dilution, amplified and tested for HVEM and pcDNA3.1 expression. Selected HVEM-expressing transfectant clones were designated as CHO-HVEM. Control, pcDNA3.1-expressing clones were designated as CHO-pcDNA. To generate transfectants stably expressing wild type or mutated HSV-1-gD, the pCJ3 and pCJ1 pcDNA3.1-based vectors (kindly provided by Prof. Patricia G. Spear, Northwestern University, Chicago, IL), encoding for the wild type gD and the gDrid mutated form of the protein, respectively, were amplified and transfected into adherent I143tk$^-$ cells. The gDrid mutated form of gD presents the substitution of glutamine 27 with proline (Q27P; Dean et al., 1995). Transfection of I143tk$^-$ cells with pCJ3 and pCJ1 or the corresponding pcDNA3.1 empty vector, was carried out as described above for cells transfected with pcDNA-HVEM. The wild type HSV-1-gD-expressing transfectants and the mutated HSV-1-gDrid-expressing transfectants were designated as I143-J3 and I143-J1, respectively. Control, empty vector expressing transfectants were designated as I143-pcDNA.

2.5. Inhibition of gD binding by neutralizing antibodies

To inhibit the interaction of gD with HVEM, recombinant gD at 1 μg/ml or UV-HSV-1 inocula were mixed with 0.1% HD1 or 1D3 MAbs and pre-incubated on ice for 1 h. As a control, mouse anti-gC 1104/R633 MAb was utilized. The gD/MAbs or UV-HSV-1/MAbs mixtures were then added to 10^7 U937 cells in 1 ml of culture medium. After 1 h of incubation at 37 °C, cells were collected and processed for further experimental procedures.

2.6. Flow cytometry

For gD detection by immunofluorescence, transfectant cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature before the addition of 0.1% Triton X-100 in PBS with 0.1% FCS for a further 5 min. After washing twice in PBS with 3% FCS, cells were incubated for 30 min with anti-gD MAb 1103/H170 on ice and washed twice in PBS. Cells were then incubated with the FITC-conjugated goat anti-mouse-IgG for a further 30 min on ice and then rinsed twice in PBS. As a control, cells were stained with the second-step reagent only. No evidence for non-specific binding to I143tk$^-$ transfectants was obtained when an isotype matched, irrelevant MAb was utilized as a control, in preliminary experiments. For HVEM detection, cells were collected, washed in PBS and pelleted. An amount of 50 μl blocking reagent (50% FCS and 50% normal goat IgG 1 mg/ml) was added to the tubes and cells were incubated 10 min on ice. Without washing, cells were then incubated with anti-HVEM ANC3B7 MAb on ice for 30 min. Cells were then washed twice in PBS and incubated with the FITC-conjugated goat anti-mouse-IgG for a further 30 min on ice and rinsed twice in PBS. In preliminary experiments, this protocol proved to completely avoid the risk for non-specific binding. Control samples consisted of cells stained with the second-step reagent only. Samples of 5000 or 10,000 cells were acquired and analysed on a Becton Dickinson FaCScalibur using the CELLQuest II software.

2.7. Cocultivation of U937 cells with stable transfectans expressing wild type or mutated gD

In cocultivation experiments, aliquots of 1×10^7 U937 growing in suspension, cells were added to cultures of adherent I143-J3, I143-J1 or I143-pcDNA transfectants, stably expressing wild type gD, mutated gD or the empty vector, respectively, at ≈80% confluence in 75 cm^2 flasks for 1 h. Supernatants containing the non-adhering cells were then gently collected, centrifuged, washed and apoptosis was then induced by addition of anti-human-Fas, to U937 cells exposed to control or gD-expressing transfectants, for 1 h at 4 °C. After a further 5 h incubation at 37 °C cells were harvested and processed for testing apoptosis.

2.8. Induction and evaluation of apoptosis

For experiments on Fas-mediated apoptosis, U937 cells were incubated for 1 h at 4 °C with vehicle alone, as a control, with infectious HSV-1, with UV-HSV-1 or with recombinant gD at the concentration of 1 μg/ml. Virus inocula were then removed and fresh growth medium was added in samples exposed to the virus. Successively, vehicle, as a control, or an agonist anti-Fas antibody were added to the cultures and cells were incubated for a further 1 h at 4 °C. Cultures were then shifted to 37 °C and apoptosis was detected in all cultures after a further 4 h incubation.

For experiments on staurosporine-induced apoptosis, U937 cells and CHO transfectants were incubated for 1 h at 37 °C with control vehicle or with UV-HSV-1, at a MOI of 100 PFU/cell. At the end of incubation time, control vehicle and staurosporine (Sigma–Aldrich), at 0.5 μM for U937 cells or at 1 μM for CHO transfectants, were added to the cells. Apoptosis was evaluated after a further 7 h incubation, for U937 cells, or 16 h incubation, for CHO transfectants. The different protocols for induction of apoptosis by staurosporine in U937 and CHO cells were chosen on the basis of preliminary experiments showing that U937 cells were more sensitive than CHO cells to this apoptosis-inducing agent.

Apoptosis in all samples was evaluated by morphological analysis of the cells following staining with acridine orange as previously described in Ref. [21]. Briefly, over 600 cells, including those showing typical apoptotic characteristics, were counted using a fluorescence microscope. The identification of apoptotic cells was based on the presence of uniformly stained nuclei showing chromatin condensation and nuclear fragmentation.

2.9. *Evaluation of caspase 3 activity*

To evaluate caspase 3 enzymatic activity, the caspase 3 assay colorimetric kit (Sigma–Aldrich) was utilized according to manufacturer's instructions. Briefly, for each experimental condition quadruplicate pellets of 5×10^5 cells were suspended in lysis buffer on ice for 20 min. After centrifugation at 16,000 × *g* for 15 min at 4 °C, the lysates were immediately processed in a 96 well plate by addition of the Ac-DEVD-CHO caspase 3 inhibitor in one of the quadruplicate samples and of the caspase 3 substrate in all the wells. After 90 min incubation at 37 °C, o.d. was read at 405 nm. The o.d. value obtained in the sample in which the caspase 3 inhibitor was added was subtracted from the o.d. values obtained in the corresponding triplicates without inhibitor. The relative caspase 3 activity was calculated as the ratio of each subtracted value to the mean o.d. value obtained in triplicate samples from cells treated with vehicle alone and results were expressed as caspase 3 activity fold induction with respect to control.

2.10. *Statistical analysis*

Data analysis was performed using the SPSS statistical software system (version 12.0 for Windows, Chicago, IL). Comparisons of means were carried out using the Tukey's honestly significance test (HSD), as a multiple comparison test.

3. Results

3.1. *Effects of treatment with infectious HSV-1, UV-HSV-1 or HSV-1-gD on Fas-induced apoptosis in U937 cells*

We have previously demonstrated that infectious HSV-1, as well as UV-HSV-1 or recombinant gD, promptly activate NF-κB in U937 cells, increasing the resistance of these cells towards apoptosis [17]. More recently, we observed that NF-κB activation by UV-HSV-1 was not restricted to U937 cells, but occurred also in other cells sharing with U937 cells HVEM-expression [20]. Conversely, non-HVEM-expressing cells were refractory to this activation, suggesting the involvement of this receptor in the observed phenomena [20]. Thus, we asked whether HVEM would be involved also in protection against apoptosis by HSV-1 and its envelope protein gD. To answer this question, based on our previous experience [17] and on preliminary experiments, we firstly determined a set of experimental conditions to better detect and compare among them, using standardized procedures, the protective effects of infectious HSV-1, UV-HSV-1 and gD alone, towards Fas-induced apoptosis in U937 cells. These conditions are described in details in Section 2. Note that actual surface expression of HVEM in our U937 cells had been recently confirmed [20] and that preference for a 4 h culture after exposure to HSV-1 or gD, differently from longer incubation time we used in previous studies [17], was motivated to limit as much as possible the possible contribution of newly expressed viral gene products, in addition to virion structural proteins, to the protective effect of infectious HSV-1 on apoptosis. Results shown in Fig. 1 indicate that, using the experimental conditions we identified, addition of anti-Fas alone induced a highly significant increase in apoptosis but a prompt protection against Fas-mediated apoptosis in U937 cells was clearly detectable following each of the three treatment utilized. Moreover, these results show that, in the identified experimental conditions, protection

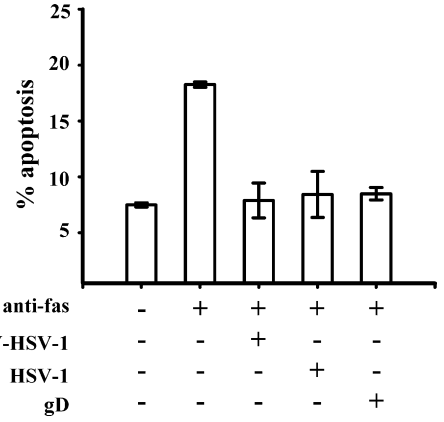
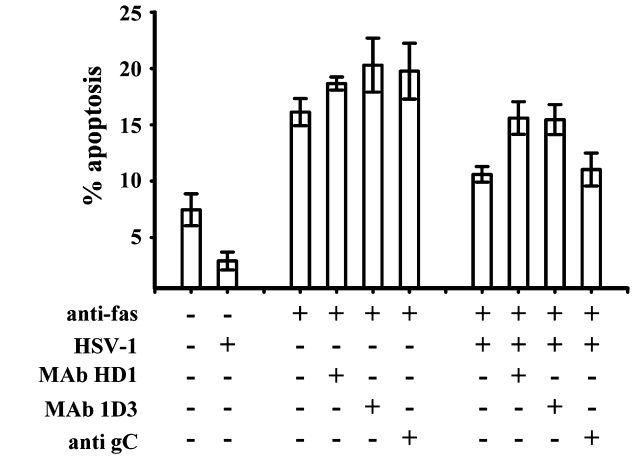


Fig. 1 – Effects of treatment with infectious HSV-1, UV-HSV-1 or HSV-1-gD on Fas-induced apoptosis in U937 cells. U937 cells were exposed to control vehicle, infectious HSV-1 (MOI = 50 PFU/cell), UV-inactivated HSV-1 (MOI = 200 PFU/cell) or soluble recombinant gD (1 μg/ml) for 1 h before addition of anti-Fas antibody (500 ng/ml) to induce apoptosis. Percentage of apoptotic cells, detected 5 h after anti-Fas addition, was evaluated using fluorescence microscopy following staining with acridine orange. As a control, samples exposed to control vehicle alone or to control vehicle plus anti-Fas were also assayed. Results, obtained from triplicate cultures, are expressed as mean values ±S.D. of one of three experiments with similar results. Multiple comparisons, by Tukey's honestly significant difference (HSD) test, were not significant among groups except for anti-Fas versus all groups ($p < 0.001$).



	vehicle	HSV	a-fas	a-fas +HD1	a-fas +1D3	a-fas +a-gC	a-fas +HSV	a-fas +HSV+HD1	a-fas +HSV+1D3
HSV	0.036	--	--	--	--	--	--	--	--
a-fas	<0.001	<0.001	--	--	--	--	--	--	--
a-fas+HD1	<0.001	<0.001	NS	--	--	--	--	--	--
a-fas+1D3	<0.001	<0.001	NS	NS	--	--	--	--	--
a-fas+a-gC	<0.001	<0.001	NS	NS	NS	--	--	--	--
a-fas+HSV	NS	<0.001	0.006	<0.001	<0.001	<0.001	--	--	--
a-fas+HSV+HD1	<0.001	<0.001	NS	NS	0.028	NS	0.017	--	--
a-fas+HSV+1D3	<0.001	<0.001	NS	NS	0.022	NS	0.021	NS	--
a-fas+HSV+a-gC	NS	<0.001	0.0014	<0.001	<0.001	<0.001	NS	0.035	0.044

Fig. 2 – Effects of inhibition of gD/HVEM interaction by MAbs on reduction of Fas-induced apoptosis by infectious HSV-1 in U937 cells. U937 cells were exposed to control vehicle or infectious HSV-1 pre-incubated for 1 h in the absence or in the presence of anti-gD HD1 or 1D3 MAbs, that block gD/HVEM interaction, or of anti-gC MAb, as a control, before the addition of anti-Fas antibody to induce apoptosis. Apoptosis, detected 5 h after anti-Fas addition, was evaluated as reported for Fig. 1. As a control, apoptosis levels in cells exposed to MAbs only and treated with anti-Fas were also assayed. Results, obtained from triplicate cultures, are expressed as mean values ±S.D. of one of two experiments with similar results. *p*-Values of all pairwise comparisons among group means, by Tukey's honestly significant difference (HSD) test, are reported in the grid under the graph (NS: not significant).

against Fas-mediated apoptosis in U937 cells exerted by infectious HSV-1 was perfectly comparable with those exerted by UV-HSV-1 or gD alone (Fig. 1). Thus, similar experimental conditions were utilized for successive investigations.

3.2. *Effects of inhibition of gD/HVEM interaction by MAbs on reduction of Fas-induced apoptosis by infectious HSV-1 in U937 cells*

We have recently observed that the capacity of gD to induce NF-κB activation was reduced in the presence of an antibody known to interfere with gD-HVEM interaction [20]. To investigate whether HSV-1-gD binding to HVEM, during the first encounter with U937 cells, could play a role in initiating the prompt anti-apoptotic effect generated by HSV-1, in the first phase of infection, firstly we exposed U937 cells to infectious HSV-1, after pre-incubation of the virus inoculum in the presence or absence of the gD-specific HD1 or 1D3 MAbs or of the 1104 MAb. HD1 and 1D3 are MAbs known to prevent binding of gD to HVEM trough recognition of specific gD epitopes. MAb 1104, that recognizes glycoprotein C, was utilized as a control. Virus inocula, pre-incubated or not with the MAbs, were then removed and apoptosis was induced by anti-Fas antibody and detected as described in Section 2. Results shown in Fig. 2 indicate that pre-incubation with both HD1 and 1D3 MAbs significantly reverted the capability of HSV-1 to inhibit anti-Fas induced apoptosis in U937 cells. Conversely, no reduction in protection against apoptosis was observed when HSV-1 was pre-incubated with MAb 1104, as a control. Addition of gD specific MAbs, as well as of the gC specific MAb, did not cause any significant modifications in apoptosis levels of U937 cells incubated with anti-Fas without exposure to HSV-1. These results indicated that the capability of binding HVEM through the envelope gD could be an important requirement for HSV-1 to induce the protection against apoptosis in HVEM-expressing cells.

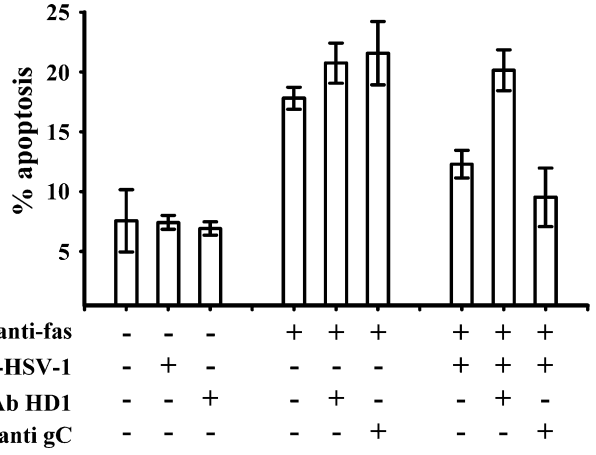
3.3. *Effects of inhibition of gD/HVEM interaction by MAbs on reduction of Fas-induced apoptosis by UV-HSV-1 in U937 cells*

Experiments using pre-incubation of infectious HSV-1 with anti-gD MAbs clearly demonstrated the importance of gD/HVEM interaction to determine the eventual protection by

HSV-1 against apoptosis in U937 cells. However they did not allowed us to distinguish whether prevention of apoptosis reduction was actually related to the capability of such a treatment to hinder the physical interaction between gD and HVEM or to interfere with the completion of the replicative cycle of HSV-1 in U937 cells. To investigate this aspect, we repeated our experiments using virus inocula inactivated by UV treatment, in place of infectious HSV-1, and the HD1 anti-gD MAb, a control anti-gC MAb or UV-treated medium, as a control vehicle, during the pre-incubation phase. Anti-Fas antibody was then added and apoptosis was assessed, as described for previous experiments. Results shown in Fig. 3, demonstrate that UV-inactivated-HSV-1 retained, as expected, its ability to inhibit apoptosis despite the fact that it had lost its capability to replicate in two independent experiments performed. Moreover, similarly to what observed for the infectious virus, pre-incubation of UV-HSV-1 with anti-gD MAb, but not with anti-gC MAb, completely abrogated the efficacy of this non-replicating virus to prevent anti-Fas induced apoptosis, while the same MAb did not affect by itself Fas-induced apoptosis (Fig. 3). From these experiments we concluded that the inhibitory action on Fas-mediated apoptosis in U937 cells was presumably due to signalling generated by HVEM after contacts with the HSV-1 structural component gD rather than by events occurring successively to HSV-1 entry and replication.

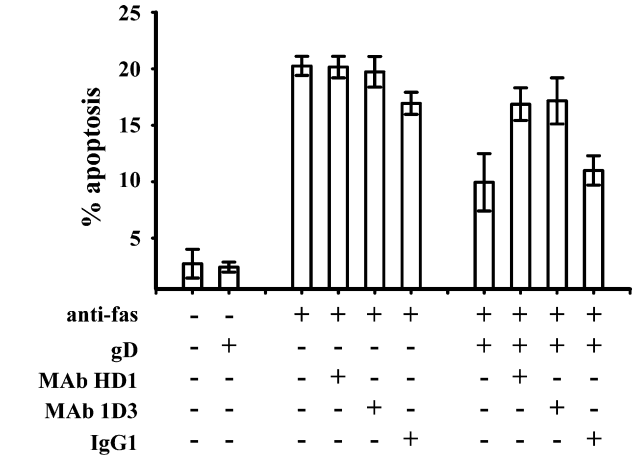
3.4. *Effects of inhibition of gD/HVEM interaction by MAbs on reduction of Fas-induced apoptosis by soluble gD in U937 cells*

To further investigate how significant the capability of gD to specifically bind HVEM was in initiating cellular signaling to prevent apoptosis, we then exposed U937 cells to control vehicle or to soluble gD, after pre-incubation of the glycoprotein in the presence or absence of HD1 and 1D3 gD-specific MAbs. An isotype IgG1 MAb was utilized as a control. After 1 h of incubation at 4 °C with control vehicle or with the gD/MAb mixtures the anti-Fas antibody was added to induce apoptosis. As a control, apoptosis level in cells exposed to MAbs only and treated with anti-Fas was also assayed. Results shown in Fig. 4 indicate that pre-incubation with anti-gD MAbs actually counteracted the inhibitory activity of recombinant gD towards Fas-mediated apoptosis in U937 cells. Conversely, no significant modification in apoptosis prevention was observed when gD was pre-incubated with the isotype control



	vehicle	UVHSV	HD1	a-fas	a-fas +HD1	a-fas +a-gC	a-fas +UVHSV	a-fas + UVHSV +HD1
UVHSV	NS	--	--	--	--	--	--	--
HD1	NS	NS	--	--	--	--	--	--
a-fas	<0.001	<0.001	<0.001	--	--	--	--	--
a-fas+HD1	<0.001	<0.001	<0.001	NS	--	--	--	--
a-fas+a-gC	<0.001	<0.001	<0.001	NS	NS	--	--	--
a-fas+UVHSV	NS	NS	0.033	0.027	<0.001	<0.001	--	--
a-fas+UVHSV+HD1	<0.001	<0.001	<0.001	NS	NS	NS	0.001	--
a-fas+UVHSV+a-gC	NS	NS	NS	0.001	<0.001	<0.001	NS	<0.001

Fig. 3 – Effects of inhibition of gD/HVEM interaction by MAbs on reduction of Fas-induced apoptosis by UV-HSV-1 in U937 cells. U937 cells were exposed to control vehicle or UV-inactivated HSV-1 pre-incubated for 1 h in the absence or in the presence of HD1 anti-gD MAb or 1104 anti-gC MAb (as a control), before the addition of anti-Fas antibody to induce apoptosis. Apoptosis, detected 5 h after anti-Fas addition, was evaluated as reported for Fig. 1. As a control, apoptosis levels in cells exposed to control vehicle, to anti-gD or anti-gC MAbs alone or to anti-Fas were also assayed. Results, obtained from triplicate cultures, are expressed as mean values ±S.D. of one of three experiments with similar results. *p*-Values of all pairwise comparisons among group means, by Tukey's honestly significant difference (HSD) test, are reported in the grid under the graph (NS: not significant).



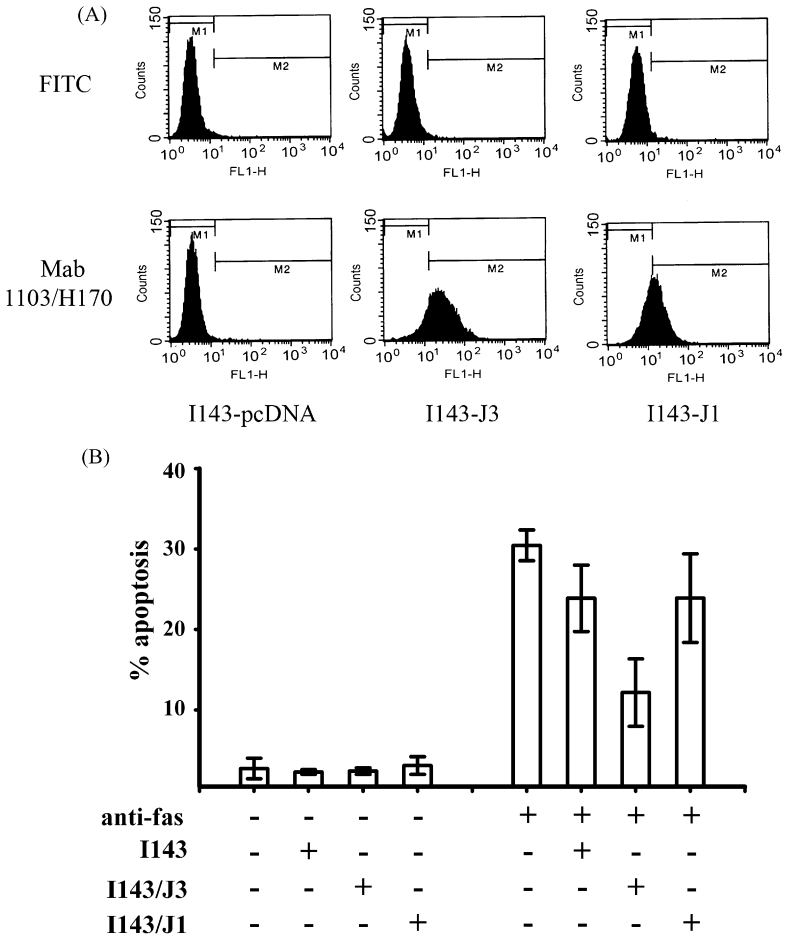
	vehicle	gD	a-fas	a-fas +HD1	a-fas +1D3	a-fas +IgG1	a-fas +gD	a-fas +gD +HD1	a-fas +gD+ 1D3
gD	NS	--	--	--	--	--	--	--	--
a-fas	<0.001	<0.001	--	--	--	--	--	--	--
a-fas+HD1	<0.001	<0.001	NS	--	--	--	--	--	--
a-fas+1D3	<0.001	<0.001	NS	NS	--	--	--	--	--
a-fas+IgG1	<0.001	<0.001	NS	NS	NS	--	--	--	--
a-fas+gD	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	--	--	--
a-fas+gD+HD1	<0.001	<0.001	NS	NS	NS	NS	<0.001	--	--
a-fas+gD+1D3	<0.001	<0.001	NS	NS	NS	NS	<0.001	NS	--
a-fas+gD+IgG1	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	NS	0.002	0.001

Fig. 4 – Effects of inhibition of gD/HVEM interaction by MAbs on reduction of Fas-induced apoptosis by soluble gD in U937 cells. U937 cells were exposed to control vehicle or recombinant HSV-1 gD pre-incubated for 1 h in the absence or in the presence of anti-gD HD1 or 1D3 MAbs or an isotype control MAb (IgG1), before addition of an agonist anti-Fas antibody to induce apoptosis. Apoptosis, detected 5 h after anti-Fas addition, was evaluated as reported for Fig. 1. As a control, apoptosis levels in cells exposed to control vehicle, gD alone, or MAbs plus anti-Fas were also assayed. Results, obtained from triplicate cultures, are expressed as mean values ±S.D. of one of two experiments with similar results. *p*-Values of all pairwise comparisons among group means, by Tukey's honestly significant difference (HSD) test, are reported in the grid under the graph (NS: not significant).

MAb (Fig. 4). Addition of MAbs only did not modify anti-Fas induced apoptosis. These results further sustained that the capability of binding HVEM could be an important requirement for HSV-1 gD to protect against apoptosis in HVEM-expressing cells.

3.5. *Effects of prevention of gD/HVEM interaction by gDrid mutation on reduction of Fas-induced apoptosis by gD in U937 cells*

Glycoprotein D of HSV-1 is expressed on the surface of cells in which HSV-1 is actively replicating. Also in this form of surface-expressed protein, gD was able to protect U937 cells against Fas-induced apoptosis [17]. To further investigate the role of gD binding to HVEM in prevention of apoptosis by HSV-1, we thought to take advantage by the existence of the *rid* mutated form of gD. This mutated form of gD, presenting amino acid substitution at position 27 [22], does not bind HVEM, as previously described in Ref. [23], but has no impaired functional interaction with other HSV-1 receptors, such as nectin-1, or even increased affinity for nectin-2 [24]. We, then, stably transfected adherent I143tk$^-$ cells with an empty vector pcDNA3.1, as a control, with a vector expressing a wild type gD that binds to HVEM and with a vector expressing the *rid* mutated form of gD. Expression of either the wild type gD (gDwt) or the mutated form of gD (gDmut) in transfectants designed as I143-J3 and I143-J1, respectively, was assessed by flow cytometry analysis following staining with 1103/H170, a gD specific MAb that recognizes an epitope mapping amino acids 11–19 [25], i.e. not the *rid* mutation site. Both I143-J3 and I143-J1 transfectants expressed the protein at surface level in most, even if not all, the cells, as detected by flow cytometry (Fig. 5A), while no expression was detected, as expected, in control, empty-vector-expressing transfectants (I143-pcDNA). A slight difference in mean florescence intensity was observed in I143-J3 and I143-J1 transfectants, probably due to a lower affinity of 1103/H170 MAb for gDmut with respect to gDwt. The generated transfectants were used for an experimental model consisting of HVEM+ target cell stimulation by cells expressing gD in the form of protein present on the cell membrane. To this purpose, we cocultured non-adherent HVEM+ U937 cells with adherent I143-J3 transfectants, with I143-J1 transfectants, with control transfectants or with vehicle



	vehicle	I143	I143/J3	I143/J1	a-fas	a-fas +I143	a-fas +I143/J3
I143	NS	--	--	--	--	--	--
I143/J3	NS	NS	--	--	--	--	--
I143/J1	NS	NS	NS	--	--	--	--
a-fas	<0.001	<0.001	<0.001	<0.001	--	--	--
a-fas+I143	<0.001	<0.001	<0.001	<0.001	NS	--	--
a-fas+I143/J3	0.020	0.014	0.015	0.028	<0.001	0.001	--
a-fas+I143/J1	<0.001	<0.001	<0.001	<0.001	NS	NS	0.001

Fig. 5 – Effects of prevention of gD/HVEM interaction by gDrid mutation on reduction of Fas-induced apoptosis by gD in U937 cells. (A) Adherent I143tk$^-$ cells were transfected with an empty vector, as a control (I143-pcDNA), or with expression vectors encoding for wild type HSV-1 gD (I143-J3) or mutated gD-rid (I143-J1) unable to bind HVEM receptor. The expression at the surface level of the two proteins was detected by flow cytometry following staining with MAb 1103/H170 and FITC goat anti-mouse IgG. Histograms in the upper panels represent staining with anti-mouse IgG only. M1 and M2 markers indicate the boundaries between negative and positive cells which were arbitrarily set for control stained samples (M2 < 0.50%) and maintained in the corresponding anti-HVEM stained samples. (B) U937 cells were cocultured for 1 h with I143-J3, I143-J1 or control I143-pcDNA adherent stable transfectants (I143). Non-adherent cells were then harvested, centrifuged and pellets were then suspended in fresh medium. Anti-Fas antibody was then added to induce apoptosis. Apoptosis was evaluated, as reported for Fig. 1, after a further 5 h incubation at 37 °C. As a control, apoptosis levels in cells exposed to adherent stable transfectants only and treated with anti-Fas was also assayed. Results, obtained from triplicate cultures for samples without anti-Fas and from quadruplicate cultures for samples with anti-Fas, are expressed as mean values ±S.D. of one of two experiments with similar results. *p*-Values of all pairwise comparisons among group means, by Tukey's honestly significant difference (HSD) test, are reported in the grid under the graph (NS: not significant).

alone for 1 h. At the end of coculture, non-adherent cells were harvested, centrifuged and incubated in the presence of vehicle or anti-Fas for a further 1 h at 4 °C. Cultures were then shifted to 37 °C and incubated for a further 5 h before apoptosis was detected. Fas-mediated apoptosis was clearly down-regulated after coculture of U937 cells with I143-J3 transfectants in comparison with cells incubated with vehicle or with control I143-pcDNA transfectants (Fig. 5B). Conversely, coculture with I143-J1 transfectants expressing the *rid* mutated, non-HVEM-binding, form of gD did not cause any significant modification in apotosis level in respect to incubation with vehicle or control transfectants (Fig. 5B). These results demonstrated that the ability to bind HVEM is a necessary requirement for membrane-anchored gD to induce protection against Fas-induced apoptosis in U937 cells.

3.6. *Effects of treatment with UV-HSV-1 on cell death and caspase 3 activity induced by staurosporine in U937 cells*

Results reported above and those reported in our previous study [17] clearly stated the capacity HSV-1, by means of its envelope gD, to inhibit the extrinsic pathway of apoptosis activated by anti-Fas linkage to Fas receptor in U937 cells. Thus, we were interested in whether a similar protective effect could be exerted by HSV-1 towards death triggered by a stimulus acting on the so-called intrinsic pathway of apoptosis, in the same cells. For this reason, U937 cells were pre-incubated for 1 h at 37 °C with control vehicle or with UV-HSV-1, at a MOI 100 PFU/cell, and at the end of incubation time, control vehicle and staurosporine were added to the cells. After a further 7 h incubation, aliquots of cells from cultures subjected to different experimental conditions were collected and apoptosis was evaluated. Moreover, parallel aliquots of cells from the same cultures were utilized for evaluation of dead and living cells, using a standard trypan blue dye exclusion test, and for assaying caspase 3 activity. Incubation with UV-HSV-1 high significantly inhibited apoptosis induced by staurosporine in U937 cells (Fig. 6A). Moreover, evaluation of dead cells by trypan blue staining gave results very similar to those obtained by detection of apoptosis (Fig. 6A). In addition, results of colorimetric detection of caspase 3 activity strongly resembled those of cell death assessment. Also in this case, preventive incubation with UV-HSV-1 high significantly inhibited induction of the enzymatic activity of the apoptotic effector protein caspase 3 by staurosporine, in U937 cells (Fig. 6B).

These results, demonstrated that UV-HSV-1 can protect U937 cells not only against death-receptor induced apoptosis, but also against apoptosis induced by another stimulus, such as that triggered by staurosporine. This extends to the intrinsic pathway of apoptosis the protective effect of UV-inactivated HSV-1. Moreover, results of trypan blue and caspase 3 assays suggest that classical apoptosis was the exclusive form of cell death involved in the observed phenomena.

3.7. *Effects of treatment with UV-HSV-1 on apoptosis induced by staurosporine in CHO transfectants expressing or not human HVEM*

Results of experiments with MAbs able to prevent binding of gD to HVEM, together with those using wild type gD and gD*rid*

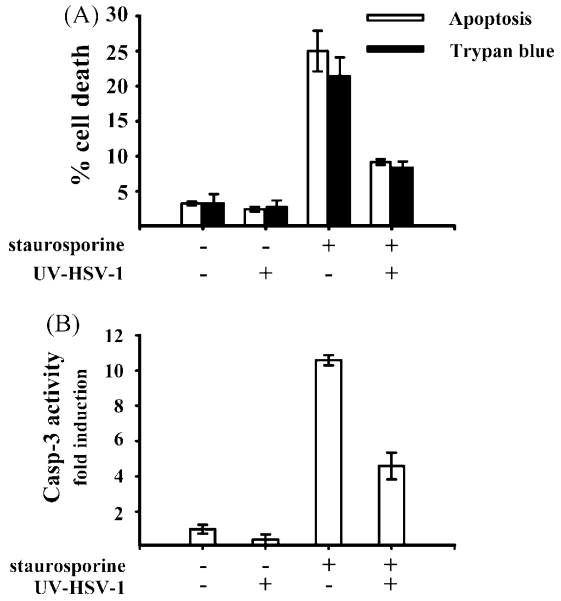


Fig. 6 – Effects of treatment with UV-HSV-1 on cell death and caspase activity induced by staurosporine in U937 cells. U937 cell cultures were exposed to control vehicle or UV-inactivated HSV-1 (MOI = 100 PFU/cell) for 1 h before addition of staurosporine (0.5 μM). After a further 7 h incubation cells were collected and processed for successive determinations. As a control, cultures exposed to control vehicle alone or to control vehicle plus staurosporine were also assayed. (A) Percentage of apoptotic dead cells (% cell death, apoptosis), was evaluated as reported for Fig. 1. In parallel, percentage of dead cells was evaluated by a standard trypan blue exclusion test (% cell death, trypan blue). Results, obtained from triplicate samples, are expressed as mean values ±S.D. Multiple comparisons, by Tukey's honestly significant difference (HSD) test, were highly significant ($p < 0.001$) among corresponding groups except for: Apoptosis, vehicle versus UV-HSV-1 (NS), vehicle versus staurosporine + UV-HSV-1 ($p = 0.005$), staurosporine versus staurosporine + UV-HSV-1 ($p = 0.002$); Trypan blue, vehicle versus UV-HSV-1 (NS), vehicle versus staurosporine + UV-HSV-1 ($p = 0.020$), staurosporine versus staurosporine + UV-HSV-1 ($p = 0.011$). (B) Caspase 3 enzymatic activity was evaluated by a colorimetric assay. The o.d. value obtained in a sample with a caspase 3 inhibitor was subtracted from each o.d. value without inhibitor. Results, obtained from triplicate samples, are expressed as mean ± S.D. caspase 3 activity fold induction with respect to control, calculated as the ratio to the mean o.d. value obtained from triplicate samples treated with vehicle alone. Multiple comparisons, by Tukey's honestly significant difference (HSD) test, were highly significant ($p < 0.001$) among corresponding groups except for: vehicle versus UV-HSV-1 (NS).

expressing transfectants, indicated that binding of gD to HVEM was a crucial requirement for inducing protection against apoptosis in U937 cells. Nevertheless, they did not allow us to exclude that, in addition to HVEM, also other cell

structures capable to bind gD could participate to protection against apoptosis by HSV-1 gD in HVEM expressing cells. To exclude this possibility, we generated transfectant clones expressing human HVEM on their surface, starting from CHO cells that are naturally resistant to HSV-1 infection and are characterized by the lack of any gD receptor that can be utilized for virus entry [26]. To generate human HVEM-expressing transfectants, the HVEM cDNA was obtained from U937 cells and inserted in a commercial expression vector under the control of the CMV promoter. Then, CHO cells were stably transfected with either the empty vector (pcDNA3.1), as a control, or with the HVEM-expressing vector. Surface expression of HVEM in stably transfected CHO cells (CHO-HVEM), but not in control transfectants (CHO-pcDNA) was confirmed by flow cytometry analysis following staining with a HVEM specific antibody (data not shown). CHO-pcDNA and CHO-HVEM transfectants were incubated for 1 h at 37 °C with control vehicle or with UV-HSV-1, at a MOI of approximately 100 PFU/cell. At the end of incubation time, control vehicle and staurosporine 1 μM, to induce apoptosis, were added to control or HVEM-expressing CHO transfectants. Apoptosis in all samples was evaluated 16 h after addition of staurosporine. CHO-HVEM transfectants showed a highly significantly reduced apoptosis level after incubation with UV-inactivated HSV-1, while apoptotic death was not modified in corresponding control transfectants after exposure to UV-HSV-1 (Table 1). Taken together with those obtained in U937 cells naturally expressing human HVEM, these results showed that protection against apoptosis by UV-HSV-1 was not cell type dependent and that expression of HVEM at cell surface was sufficient, by itself, to render sensitive to protection against apoptosis by non-replicating UV-HSV-1 cells that were not sensitive to this protective stimulus. From these experiments we conclude that signaling triggered by binding of gD to HVEM plays a crucial role in inhibition of apoptosis determined by HSV-1-gD in HVEM expressing cells.

Table 1 – Effect of exposure to UV-inactivated HSV-1 on staurosporine-induced apoptosis in CHO transfectants expressing or not human HVEM[a]

Treatment group	% apoptosis (mean ± S.D.)	
	CHO-pcDNA	CHO-HVEM
Vehicle	0.77 ± 0.25[b]	0.70 ± 0.30
UV-HSV-1	1.17 ± 0.77	0.87 ± 0.23
Staurosporine	24.40 ± 2.35	21.10 ± 0.96
Staurosporine + UV-HSV-1	23.33 ± 1.15	10.40 ± 0.53

[a] CHO cells stably transfected with a control vector (CHO-pcDNA) or with a human-HVEM-expressing vector (CHO-HVEM) were exposed for 1 h to control UV-treated vehicle or to ≈100 PFU/cell UV-inactivated-HSV-1. At the end of incubation time, staurosporine (1 μg/ml) was added and apoptosis values were detected after a further 16 h incubation.

[b] Values obtained from triplicate cultures in one of three experiments with similar results. Multiple comparisons, by Tukey's HSD test, were highly significant ($p < 0.001$) among treatment groups except for: CHO-pcDNA, vehicle versus UV-HSV-1 (NS); CHO-HVEM, vehicle versus UV-HSV-1 (NS), staurosporine versus staurosporine + UV-HSV-1 (NS).

4. Discussion

The cell receptor HVEM has been recognized as an important player in the network of signals that regulates, positively and negatively, immune cell activation by means of inter-cellular interactions through cell-surface anchored or soluble molecules with ligand/receptor functions (reviewed in [27–29]). Interestingly, HVEM has been shown to engage a plethora of natural ligands belonging to different families of molecules, including lymphotoxin-a, LIGHT, BTLA and, as recently reported [30], the glycosylphosphatidylinositol-anchored member of the immunoglobulin superfamily CD160. Not only HVEM shows the unusual characteristic of binding various molecules, but also has the unique capability to produce both a positive and a negative outcome in cell activation, depending on the engagement of the specific ligand. Thus, LIGHT acts as a costimulatory protein after engagement of HVEM, while BTLA and CD160 deliver an inhibitory signal following binding with the same receptor [27–30]. HVEM-mediated inhibitory activity affects different subsets of lymphocytes [27–30] and also dendritic cells (DCs), as recently demonstrated [31]. Glycoprotein D of HSV-1 was identified as a HVEM ligand before its natural ligands were found [18,19]. However, no signaling activity was associated with gD/HVEM interaction until recently [20]. Here we show that engagement of HSV-1 to HVEM, through its envelope gD, not only deserves virus entry, but, similarly to what occurs following engagement with its natural ligands, also induces a functional effect in cells that express this receptor.

Particularly, the functional effect exerted by gD towards HVEM-expressing cells is a pro-survival activity. In fact, we have demonstrated, using two different experimental approach such as blocking MAbs and expression of mutated gD, that when HSV-1 gD binds HVEM, U937 cells were protected from Fas-induced apoptosis but, conversely, when gD cannot bind HVEM this protective action was abrogated. Moreover, in the present paper we also demonstrated that non-replicating, UV-inactivated HSV-1 protected against apoptosis only cells in which human HVEM was expressed by stable transfection and not control transfectants not expressing this receptor. This further sustains our novel finding that HVEM engagement by HSV-1 produces by itself also an important biological effect, such as cell survival, rather than exclusively allowing virus entry. Interestingly, this biological effect could be exerted independently on the capability of the virus to infect target cells and to replicate within them, and could, thus, affect also "bystander" cells. Moreover, our results using U937 cells and HVEM-expressing CHO transfectants show for the first time that protection against apoptosis by UV-inactivated HSV-1 is not limited to death-receptor induced cell death, but also against apoptosis induced by a different stimulus, such as pharmacological induction of apoptosis by staurosporine. This is in agreement with our previous studies on mechanisms involved in protection against apoptosis by gD, showing that this phenomenon was dependent on NF-kB activation and was associated with the up-regulation of some anti-apoptotic genes that are known to protect also from death-receptor unrelated pro-apoptotic stimuli [21].

Taken together, our results suggest that signaling triggered by binding of gD to HVEM could have an important, previously

unrecognized impact towards cells of the immune compartment that are known to high express this receptor, such as lymphocytes, monocytes and DC, irrespective of the capability of HSV-1 to efficiently infect them. As a consequence, the observed phenomenon could represent an additional mechanism of evasion from premature apoptotic death exerted by HSV-1-gD in HVEM-expressing cells. In addition, demonstration that binding of gD to HVEM can prevent apoptosis discloses new opportunities of cell death manipulation by using gD preparations as a new biotechnological tool.

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