Caspase 9 Is Essential for Herpes Simplex Virus Type 2-Induced Apoptosis in T Cells

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Herpes simplex virus type 2 (HSV-2) induces apoptosis in T cells by a caspase-dependent mechanism. Apoptosis can occur via extrinsic (death receptor) and/or intrinsic (mitochondrial) pathways. Here, we show that the initiator caspase for the intrinsic pathway is activated in T cells following HSV-2 exposure. To determine the respective contributions of intrinsic and extrinsic pathways, we assessed apoptosis in Jurkat cells that are deficient in caspase 8 or Fas-associating protein with death domain (FADD) for the extrinsic pathway and in cells deficient in caspase 9 for the intrinsic pathway. Our results indicate HSV-2-induced apoptosis in T cells occurs via the intrinsic pathway.

Herpes simplex virus (HSV) reactivation in human results in accumulation and persistence of virus-specific CD4+ and CD8+ cells at the site of reactivation (15, 16). Despite such immune responses, virus reactivation can occur on >75% of days for some individuals (14). Reactivation of the virus in the midst of continued cell-mediated immunity demonstrates the ability of the virus to circumvent the host’s immune system. HSV antigens were readily detected from T cells isolated from human HSV lesions, indicating that T cells are infected with HSV in vivo (1). In vitro, HSV-infected T cells undergo apoptosis (5–7, 10), and we have additionally demonstrated that apoptosis occurs in Jurkat cells, a T-cell leukemia line, and primary CD4+ T lymphocytes isolated from human peripheral blood mononuclear cells following exposure to HSV type 2 (HSV-2)-infected human foreskin fibroblasts (5). These results suggest that induction of T-cell death is a mechanism by which the virus limits the effectiveness of local cell-mediated immunity during reactivation.

Since T cells are most likely exposed to HSV-2 via infected epithelial cells in vivo, we examined T-cell apoptosis following exposure to infected fibroblasts in vitro. To evaluate whether HSV-2-exposed primary T cells undergo apoptosis by a caspase-dependent mechanism, we examined activation of caspase 3 in human CD4+ cells after exposure to HSV-2-infected fibroblasts. Human foreskin fibroblasts (American Type Culture Collection, Manassas, VA) were mock infected or infected with the HSV-2 HG52 strain at a multiplicity of infection of 5. At 6 h postinfection, CD4+ cells were exposed to mock-infected or HSV-2-infected fibroblasts at a ratio of approximately 3:1 (lymphocytes to fibroblasts). CD4+ cells were isolated to >95% purity using MACS CD4 microbeads (Miltenyi Biotec, Inc., Auburn, CA) immediately prior to experiments with human peripheral mononuclear cells (Memorial Blood Centers, St. Paul, MN) that were stimulated with phytohemagglutinin (Sigma Aldrich, St. Louis, MO) for 48 h and then maintained with interleukin-2 (Invitrogen, Carlsbad, CA) as previously described (5). After 4 h of incubation, CD4+ cells were harvested and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 0.3 ng/ml interleukin-2. At 24 h and 48 h postexposure, CD4+ cells were probed with a fluorescence-labeled antibody against activated caspase 3 (BD Biosciences, San Jose, CA). Data were collected on a FACSCanto (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). Side scatter and fluorescence were used as dual parameters to clearly gate the cell population with activated caspase 3, and an identical gate was applied to all samples within each experiment. As shown in Fig. 1, the percentage of cells with activated caspase 3 was 23% in HSV-2-exposed CD4+ cells compared with 5% in mock-exposed cells at 24 h postexposure. At 48 h, the percentage of cells with activated caspase 3 increased to 39% in HSV-2-exposed cells compared to 8% in mock-exposed cells. These percentages are comparable to those for Jurkat cells in our previous report (19% for virus-exposed cells versus 5% for mock-exposed cells at 24 h postexposure) (5).

Efficient infection of T cells by HSV upon exposure to HSV-infected human fibroblasts via cell-to-cell spread has been recently demonstrated (1). To delineate the relationship between virus infection and apoptosis, Jurkat cells were exposed to mock-infected or HSV-2-infected fibroblasts as described above and then coprobed with antibodies for activated caspase 3 and HSV-2 ICP10. As shown in Fig. 2, two antibodies demonstrated largely mutually exclusive populations in cells exposed to HSV-2-infected fibroblasts. We obtained similar results with antibodies against other HSV-2 antigens, including glycoprotein B, ICP5, and ICP8 (data not shown). The results suggest that infected cells may be inducing apoptosis in uninfected cells via a bystander effect, similar to what has been proposed for HSV-1-infected activated cytotoxic T cells (11). Alternatively, the activation of apoptosis may result in degradation of viral antigens in infected cells.

Our results demonstrate an activation of caspase 3 in HSV-2-exposed T cells, suggesting a caspase-mediated apoptosis. Previous studies have supported such a mechanism, as caspase inhibitors block virus-induced cell death (5, 10). Caspase-de-
Apoptosis can occur via extrinsic (death receptor) and/or intrinsic (mitochondrial) pathways (3). Here, we sought to determine the respective contribution of each pathway in HSV-2-induced T-cell death.

To investigate the contribution of individual apoptosis pathways in HSV-2-exposed T cells, we first evaluated mitochondrial membrane potential in CD4+ and Jurkat cells following virus exposure. Mitochondria play a major role in the intrinsic apoptosis pathway (2, 3). T cells were exposed to mock-infected or HSV-2-infected fibroblasts as described above and then probed with chloromethyl-X-rosamine (CMXRos; Invitrogen) at 24 h and 48 h postexposure. CMXRos is a lipophilic cationic fluorescent dye whose staining of cells is dependent on negative mitochondrial membrane potential, and a loss of staining indicates a loss of mitochondrial potential (2, 9). As shown in Fig. 3, a loss of mitochondrial membrane potential was observed in increased percentages of HSV-2-infected fibroblasts.
exposed CD4+ and Jurkat cells compared to those for mock-exposed cells. The percentage of cells with a loss of mitochondrial membrane potential reached 49% for CD4+ cells and 92% for Jurkat cells at 48 h postexposure. The changes in mitochondrial membrane potential in HSV-2-exposed T cells suggest that the intrinsic apoptosis pathway is activated in these cells. We previously reported that Jurkat and CD4+ cells were equally susceptible to apoptosis induced by anti-Fas monoclonal antibody (5). A recent report suggests that activated primary T cells are less susceptible to HSV infection than are Jurkat cells (1). Thus, we believe that a lower percentage of CD4+ cells with a loss of mitochondrial membrane potential than Jurkat cells is due to lower susceptibility to HSV infection.

To further define the involvement of intrinsic and extrinsic apoptosis pathways in HSV-2-induced T-cell apoptosis, we investigated whether caspase 8, an initiator protease for the extrinsic pathway, and caspase 9, an initiator protease for the intrinsic pathway, are activated in HSV-2-exposed T cells. CD4+ and Jurkat cells were exposed to mock-infected or HSV-2-infected fibroblasts as described above. Cell lysates were made at 24 h postexposure, and immunoblots were performed with 30 to 50 μg of protein per well, as described previously (5), with anti-caspase 8 and anti-caspase 9 antibodies (Cell Signaling, Danvers, MA). A 24-h time point was chosen for the immunoblots in order to minimize the detection of baseline apoptotic activity that can be seen with primary CD4+ cells in cultures with a longer incubation time.

As shown in Fig. 4, CD4+ cells that were exposed to HSV-2 had an increase in cleaved, activated fragments of caspase 9. No clear differences between mock-infected and HSV-2-exposed CD4+ cells were seen for caspase 8 cleavage. In contrast, increases in both cleaved caspase 8 and cleaved caspase 9 were evident in Jurkat cells following virus exposure. Together, these results indicate that caspase 9 is activated in HSV-2-exposed T cells and suggest activation of the intrinsic apoptosis pathway. Although activation of caspase 8 was seen with Jurkat cells, the contribution of the extrinsic pathway remained in doubt for virus-induced apoptosis in CD4+ cells.

To determine the respective contributions of each pathway, we assessed apoptosis in Jurkat cells that are deficient in either caspase 8 or Fas-associating protein with death domain (FADD) for the extrinsic pathway and in cells deficient in caspase 9 for the intrinsic pathway. We chose caspase 3 activation to assess apoptosis in HSV-2-exposed cells, because
convergence of extrinsic and intrinsic apoptosis pathways occurs at caspase 3.

Jurkat cells deficient in caspase 8 or FADD (American Type Culture Collection) have been previously described (8), and we confirmed the absence of caspase 8 and FADD in respective cell lines (Fig. 5A). We also investigated the parental Jurkat cell A3 subclone and verified that both caspase 8 and FADD were expressed as expected (Fig. 5A). Parental and deficient Jurkat cells were exposed to mock-infected or HSV-2-infected fibroblasts as described above. At 24 h postexposure, cells were probed with fluorescence-labeled antibody against activated caspase 3. As shown in Fig. 5B, cells deficient in caspase 8 or FADD underwent apoptosis at levels similar to those of parental cells as determined by caspase 3 activation. The results indicate that neither caspase 8 nor FADD is required for HSV-2-induced apoptosis in Jurkat cells. We additionally evaluated for cleavage products of caspase 8 and caspase 9 in these cells. As shown in Fig. 5C, cleaved fragments of caspase 9 were seen in all three cell types following HSV-2 exposure. Interestingly, cleaved fragments of caspase 8 were detected in both parental and FADD-deficient cells, suggesting that FADD is not required for cleavage of caspase 8 following HSV-2 exposure.

Jurkat cells deficient in caspase 9 expression and the corresponding caspase 9-reconstituted clones (12, 13) were a gift of Ingo Schmitz (University of Dusseldorf, Dusseldorf, Germany). Immunoblot analysis confirmed the presence or absence of caspase 9 in both cell lines (Fig. 6A). Cells were exposed to mock- or HSV-2-infected fibroblasts and analyzed for activation of caspase 3 as described above. Following exposure to HSV-2, cells deficient in caspase 9 were protected from apoptosis as determined by caspase 3 activation (Fig. 6B). In contrast, cells with reconstituted caspase 9 showed an increased percentage of caspase 3 activation. An evaluation of caspase 8 and caspase 9 cleavage in these cells showed that cleaved fragments were seen only in the cells reconstituted with caspase 9 (Fig. 6C). To confirm that the findings were applicable to the parental Jurkat cell line, we evaluated the effect of a caspase 9 inhibitor, Z-LEHD-fmk (R&D Biosystems, Minneapolis, MN) on Jurkat cells that were exposed to mock- or HSV-2-infected fibroblasts. Jurkat cells were incubated with 100 μM Z-LEHD-fmk or a dimethyl sulfoxide (DMSO) solvent control for 1 h before exposure to fibroblasts, throughout the exposure, and postexposure. At 24 h postexposure, the percentage of virus-exposed cells with activated caspase 3 was inhibited by Z-LEHD-fmk to 3%, identical to the baseline percentage seen with mock-exposed cells with DMSO solvent control (Fig. 6D). Together, these findings indicate that caspase 9 is required for HSV-2-induced apoptosis and cleavage of both caspase 8 and caspase 9 in Jurkat cells.
Our results provide evidence that exposure to HSV-2-infected fibroblasts leads to apoptosis in T cells. It is important to note that our method of activation and maintenance of human peripheral blood mononuclear cells enriches for CD4+ cells. Phytohemagglutinin and interleukin-2 nonspecifically activate these cells, and no specific immune response to HSV-2 is expected. Further studies are necessary to determine if our observations with human CD4+ cells are applicable to other primary lymphocyte cell types and to virus-specific immune cells. Despite these limitations, the similarity in the pattern of change in apoptotic markers in Jurkat and primary human CD4+ cells suggests that apoptosis occurs by a similar mechanism in the two cell types.

It is our prediction that the ability of HSV-2 to kill T cells plays a role in mitigating the cell-mediated immune response in vivo. In the present study, we have begun to elucidate the mechanism behind apoptosis in T cells following exposure to HSV-2-infected fibroblasts. Viral antigens could be detected in virus-exposed Jurkat cells, suggesting infection that occurs by cell-to-cell spread. We previously reported that expression of HSV-1 ICP0 and HSV-2 ICP10 results in apoptosis of transfected Jurkat cells (4). Therefore, we predicted that infected cells would be positive for apoptotic markers in the present study. However, viral antigens and activated caspase 3 were largely mutually exclusive in virus-exposed cells. We are currently undertaking studies to further decipher the relationship between viral infection and apoptosis to determine whether apoptosis occurs by a bystander effect in T cells.

Our results revealed the requirement of caspase 9 in HSV-2-induced apoptosis of Jurkat cells. The finding that neither caspase 8 nor FADD was required suggests that the extrinsic pathway is not involved in HSV-2-induced apoptosis in Jurkat cells. Consistent with this finding, cleavage of caspase 9 was clearly demonstrated in virus-exposed CD4+ cells compared to mock-exposed cells, while cleavage of caspase 8 was not evident. Although similarities between HSV-2-induced apoptosis in CD4+ and Jurkat cells predict that similar mechanisms are likely to be responsible in both cell types, the applicability of the finding to other types of T cells remains to be elucidated.

In conclusion, the present study demonstrated activation of intrinsic apoptosis pathways in Jurkat and primary human CD4+ cells following exposure to HSV-2. Further understanding of virus-induced apoptosis in T cells, which is predicted to be an immune-evasion mechanism during reactivation, is expected to provide means to prevent HSV-2 reactivation in infected individuals.

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