Central Nervous System Apoptosis in Human Herpes Simplex Virus and Cytomegalovirus Encephalitis

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Central nervous system (CNS) specimens from 10 immunocompetent patients with herpes simplex encephalitis (HSE) and 3 infants with congenital cytomegalovirus (CMV) encephalitis were analyzed to determine whether apoptosis is a feature of CNS injury in these patients. Apoptotic neurons and glia were detected in significant numbers in acute HSE and CMV encephalitis. Occurring predominantly in areas of productive viral infection, apoptosis appeared to result from direct viral injury to neurons and was not dependent on inflammatory T cell responses. In contrast to patients with acute cases, patients with late sequelae of HSE or CMV had no detectable virus and minimal neuronal or glial apoptosis, regardless of the degree of inflammation. This is the first demonstration of apoptotic neuronal death in humans with HSE. These results suggest that neuronal apoptosis is an important contributing factor to acute CNS injury and may serve as a novel therapeutic target in these patients.

Apoptosis is a distinct mechanism of cellular death which has been increasingly implicated in the pathogenesis of a wide variety of acute and chronic neurological diseases, including stroke, epilepsy, traumatic brain injury, and neurodegenerative diseases [1–3]. Apoptosis is also an important virus-induced mechanism of neuronal injury and death in infected neuronal cell cultures in vitro and in experimental models of viral central nervous system (CNS) in vivo infection [4, 5]. Among neurotropic viruses that have been shown to induce neuronal and/or glial apoptosis are arboviruses [6–13], bornaviruses [14], herpesviruses [15], lentiviruses [16, 17], paramyxoviruses [18–20], reoviruses [21], picornaviruses [22], rhabdoviruses [23–25], and togaviruses [26–32]. Despite the wealth of evidence supporting the importance of apoptotic cell death in experimental systems, its importance as a mechanism of cellular and tissue injury during human CNS viral infection remains poorly understood.

Studies of virus-induced apoptosis in the human CNS has largely been limited to evaluation of immunodeficient patients with human immunodeficiency virus (HIV)–associated neurological deficits [33–42]. To our knowledge, the only other viral infection in which apoptosis has been evaluated in human CNS tissues is in patients with subacute sclerosing panencephalitis (SSPE) following measles virus infection [43]. Although virus has been directly implicated in neuronal and/or glial apoptotic death in patients with HIV or SSPE, these findings are not clearly applicable to the more common situation of acute encephalitides affecting immunocompetent hosts, because, in both HIV-associated CNS disease and SSPE, it is difficult to separate the impact of an altered immune response from the direct effects of acute viral infection on neuronal and/or glial cells.

The focus of our study was to investigate whether apoptosis is an important mechanism of CNS damage in immunocompetent patients suffering from herpes simplex virus (HSV) encephalitis and infants with congenital cytomegalovirus (CMV) encephalitis, 2 of the most common causes of acute viral encephalitis in nonimmunocompromised hosts. Herpes simplex encephalitis (HSE) has classically been described as a necrotizing process; however, this designation antedated understanding of the distinction between necrotic and apoptotic cellular death. Significant advances in our understanding of the pathogenesis of this disease have occurred in the past decade, but, despite the availability of acyclovir therapy, clinical outcomes from HSE are still suboptimal, with 20% mortality and 40% prevalence of resultant neurologic deficits in affected patients [44]. Multiple investigators have confirmed the apoptosis-modulating effects (both pro- and antiapoptotic) of HSV infection and gene products in various cell lines in vitro [45–52], including neurons [53, 54]. Neuronal apoptosis has also been shown to

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Human experimentation guidelines of the institutional review board of the University of Colorado Health Sciences Center were followed in the conduct of this study.

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occur in HSV-infected animals [15, 55], but the role of apoptosis in HSV-induced CNS damage in humans remains unknown.

CMV infection is the most common congenital infection affecting humans, creating a risk of severe injury to the infected fetus [56]. Up to 10% of infants born to mothers with primary CMV infection are symptomatic at birth: 10% of symptomatic infants die, and 80% of survivors suffer severe neurological morbidity (9000 infants each year in the United States) [57, 58]. CMV encephalitis is also an important opportunistic infection in HIV-infected patients but is rarely recognized in immunocompetent adults and older children, whose immune systems are more developed than that of newborn infants [59]. Pathologically, encephalitis occurs in a periventricular pattern and may further cause polymicrogyria and hydrocephalus. Multiple investigators have identified both pro- and antiapoptotic effects of CMV infection and gene products in a variety of cell lines in vitro [60–67], which include neurons [68, 69]. The study of murine CMV infection in the developing mouse brain in vivo has revealed apoptosis in microglia/macrophages and uninfected cells to a greater degree than in infected cells [70], but a murine model of CMV retinitis has revealed significant apoptosis of both infected and uninfected cells [71]. A study of apoptosis in humans with CMV infection has been limited to HIV patients with CMV retinitis, in which apoptosis was found to contribute significantly to retinal cell loss [72]. There have been no attempts to determine the role of apoptosis in the CNS damage characteristic of infants suffering from congenital CMV CNS infection. This study is the first demonstration of CNS apoptosis in patients with acute HSE and congenital CMV infection.

**Subjects, Materials, and Methods**

**Specimen Selection and Patient Description**

**Case subject.** Archived autopsy and surgical biopsy CNS tissues were collected from case subjects with human HSV and CMV encephalitis in immunocompetent hosts from University of Colorado Health Sciences Center–affiliated hospitals. Case subjects were carefully chosen to include only those in which a definitive diagnosis of specific viral infection had been previously confirmed by culture, polymerase chain reaction (PCR), or detection of viral antigen in brain tissue. In some cases, supportive serologic studies or culture of virus from other sites were available. Only well-studied case subjects with definitive diagnoses were included for analysis in this study. Furthermore, only case subjects for which adequate amounts of tissue remained for serial sectioning, as described below, and with adequate preservation for immunohistochemical analysis were included.

The majority of acute HSV cases occurred in children and young adults with no known underlying disease or other predisposing factor. One case occurred in an individual being treated with combination drug therapy for atypical mycobacterial pulmonary infection. Although no specific immunologic deficit could be identified in this patient, the neuropathological features were similar to those reported in HSV-infected immunocompromised individuals. In these patients, it has been noted that there is a lack of inflammatory cell response and widespread pseudoischemic neuronal changes in association with large numbers of viral bearing cells [73]. We chose to include this case because it provided a rare opportunity to assess apoptosis in the absence of an inflammatory response.

The CMV cases analyzed were all congenital CMV infections, since immunocompetent adults are only rarely affected [59]. CMV infections in immunocompromised patients, including those with underlying HIV infection or malignancy, were specifically excluded.

In addition to selecting patients with acute HSV and CMV CNS infection, we also wished to analyze patients with neurologic sequelae occurring months to years after severe CNS herpetic infection, to assess the potential persistence of apoptotic injury. Three such patients with remote HSV infection were identified, with CNS abnormalities consisting of severe neuronal loss, gliosis, and cavitation in bilateral temporal lobes, as well as varying degrees of persistent lymphocytic inflammation. Two patients were identified with subacute and remote CMV infection (weeks to years following acquisition of virus), both of whom had severe gyral anomalies. Demographic and clinical information for these patients is summarized in table 1.

**Exclusions.** Cases in immunocompromised individuals were specifically excluded, including those in which the patient had 1 of the 3 most common immunocompromising conditions: congenital immunodeficiency, underlying malignancy, or HIV infection. We felt that it was particularly important to exclude HIV-infected patients with secondary herpetic infection, since it would be difficult to discern the specific contribution of each virus in the pathogenic process. Case subjects were also rejected because of inadequacy of tissue samples available for sequential sectioning or tissues with compromised integrity that precluded immunohistochemical analysis.

**Control cases.** Control cases, as described above, included patients in whom infection was clearly implicated by the above criteria but in whom infection was remote at the time of disease diagnosis. Additional control cases included assessment of baseline neuronal apoptosis in normal autopsy brain tissues (normal hippocampus) from patients at various ages of neurodevelopment (infancy to late adulthood). This was important for interpretation of the significance of occasional apoptotic neuronal/glial cells noted in some remotely-infected patients, as well as for determination of baseline age/developmental stage-specific neuronal/glial apoptosis levels in uninfected patients.

**Immunohistochemical Evaluation**

A protocol of serial sectioning was undertaken from each tissue block, so that mirror-image sections could be assessed for specific virus, presence of apoptosis, and presence of inflammatory T cells. Consecutive sections were cut (4 μm thick) from formalin-fixed, paraffin-embedded tissue blocks. When available, multiple sections (up to 5) from a given case patient were analyzed as a further experimental control, including areas both histologically involved and uninvolved in the infectious process. Sections were deparaffinized and rehydrated using standard methods and then evaluated...
Table 1. Demographic and clinical summary of patients with herpes simplex virus (HSV) and cytomegalovirus (CMV) central nervous system infection.

<table>
<thead>
<tr>
<th>Virus, disease stage, patient</th>
<th>Age, sex</th>
<th>Clinical summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV Acute</td>
<td>1 19 months, M</td>
<td>Seizures and coma (encephalitis)</td>
</tr>
<tr>
<td></td>
<td>2 8 years, M</td>
<td>Encephalitis</td>
</tr>
<tr>
<td></td>
<td>3 12 years, F</td>
<td>Fever, seizures 27 days after traumatic brain injury</td>
</tr>
<tr>
<td></td>
<td>4 17 years, M</td>
<td>Meningoencephalitis</td>
</tr>
<tr>
<td></td>
<td>5 19 years, M</td>
<td>Right temporal lobe mass and edema with near-herniation</td>
</tr>
<tr>
<td></td>
<td>6 66 years, M</td>
<td>Fever, altered mental status, focal and generalized seizures, focal neurologic findings after 10-year history of pulmonary atypical mycobacteria disease while receiving chronic medication regimen</td>
</tr>
<tr>
<td></td>
<td>7 72 years, F</td>
<td>Edematous right hemisphere after <em>Escherichia coli</em> sepsis</td>
</tr>
<tr>
<td>Remote</td>
<td>8 25 years, F</td>
<td>Persistent seizure disorder and hemiparesis (HSE 12 years before)</td>
</tr>
<tr>
<td></td>
<td>9 68 years, F</td>
<td>Postherpetic neurologic deficits, language and memory deficits, confusion, gait disturbance (HSE 1 year before)</td>
</tr>
<tr>
<td></td>
<td>10 85 years, F</td>
<td>Postherpetic encephalitis dementia, aggressive behavior (HSE 3 years before)</td>
</tr>
<tr>
<td>CMV Acute</td>
<td>11 25-week EGA fetus, M</td>
<td>Congenital CMV infection with oligohydramnios, fetal ascites, hydrocephalus, multiple intracranial hemorrhages first noted 1 week prior to TAB</td>
</tr>
<tr>
<td>Subacute</td>
<td>12 23-week EGA fetus, F</td>
<td>Congenital CMV infection with oligohydramnios, fetal ascites, hepatomegaly with extramedullary hematopoesis, and polymicrogyria; high maternal CMV IgG with negative IgM, suggesting infection approximately 6–8 weeks prior to testing</td>
</tr>
<tr>
<td>Remote</td>
<td>13 3 years, F</td>
<td>Previous congenital CMV infection with resultant hydrocephalus, left hemispheric atrophy, micropolygyria, profound retardation, deafness, blindness, spastic quadriplegia; symptomatic since 1 day of age</td>
</tr>
</tbody>
</table>

NOTE. EGA, estimated gestational age; HSE, herpes simplex encephalitis; TAB, therapeutic abortion.

by the following techniques by at least 2 independent observers in a blinded fashion.

*Hematoxylin-eosin (HE) stain.* A standard protocol was utilized for general histological definition, evidence of viral inclusion bodies (Cowdry A), inflammatory T cell infiltrate, and anoxic damage.

*Virus-specific immunohistochemistry.* We analyzed all sections for the presence and distribution of specific productive viral infection. After antigen retrieval (sections microwaved for 4 min in citrate buffer) and blockade of endogenous peroxidase activity (3% *H₂O₂* for 4 min at 37°C, citrate buffer) and blockade of endogenous peroxidase activity (3% *H₂O₂* for 4 min at 37°C, citrate buffer) and blockade of endogenous peroxidase activity (3% *H₂O₂* for 4 min at 37°C, citrate buffer), sections were incubated in either HSV-specific antibody which recognizes HSV-1 and -2 (B0114/B0116, DAKO; 1:800 dilution, 32 min at 37°C) or CMV-specific antibody (MAB-012D, BioMed; prediluted, 32 min at 37°C). Sections were probed with avidin-horseradish peroxidase (Avidin-HRPO, Ventana Medical Systems). We visualized labeling by use of diaminobenzidine peroxidase substrate (Ventana Medical Systems) and counterstained using Gill’s #2 (1:9 dilution for 4 min at 37°C). All reactions were performed on a Ventana Automated Staining System. Positive and negative control cases were included for all reactions.

*Activated caspase-3/CD3 (pan-T-cell marker) immunohistochemistry (double labeling).* To simultaneously identify inflammatory T cells within sections and determine whether these were a separate or identical population of cells showing evidence of apoptosis, we performed immunohistochemical labeling on all sections using antibody directed against CD3, a pan–T-cell marker (A0452, DAKO), in conjunction with antibody directed against cleaved (activated) caspase-3 (Asp-175; Cell Signaling Technology). Caspase-3 is a cysteine protease that plays a central role in apoptosis. The presence of activated caspase-3 is a sensitive and specific marker of apoptosis. After antigen unmasking and blockade of endogenous peroxidase activity, sections were blocked and then incubated in cleaved caspase-3 antibody (1:25 dilution in blocking solution for 32 min at 37°C). Biotinylated universal antibody (Ventana) was used as secondary antiserum (8 min at 37°C). Sections were probed with avidin-HRPO (Ventana; 8 min at 37°C). We visualized activated caspase-3 labeling using diaminobenzidine peroxidase substrate (DAB; Ventana). Immediately after this step, sections were incubated with CD3 antibody (1:100 dilution; 32 min at 37°C). Biotinylated universal antibody (Ventana) was used as secondary antiserum (8 min at 37°C). We visualized labeling by CD3 antibody using the Enhanced Alkaline-phosphatase red kit (Ventana). Sections were counterstained with hematoxylin. All steps were performed on a Ventana Automated Staining System. Positive and negative control cases were used for all reactions.

*TUNEL.* We analyzed all sections for evidence of DNA fragmentation consistent with apoptotic cleavage by using in situ TUNEL (NeuroTacs II system; Trevigen). Terminal deoxynucleotidyl transferase (TdT) was utilized to incorporate biotinylated nucleotides at the sites of DNA breaks, which are characteristic of apoptosis. We made permeable sections with Proteinase K at a 1:400 dilution, diluted in a 50:50 mixture of PBS and Neuropore for 20 min at 37°C. Following the TdT-enzyme dependent step (1 h incubation at 37°C using coverslips and a humidified chamber), we treated sections with streptavidin-HRP for 20 minutes and visualized with DAB for 1–5 min. Blue counterstain was utilized to effectively visualize background cellular architecture. We performed a TdT-enzyme negative control case on all TUNEL runs to ensure that resultant brown staining was truly indicative of being TdT-dependent, rather than nonspecific background labeling.
Technical Issues/Control Cases

To ensure accurate interpretation of all immunohistochemical analyses described above, multiple negative controls were included in conjunction with all staining procedures for each evaluated case. Negative control sections were treated identically to sections for analysis within the same run, with the exception of the key enzyme or antibody required for specific immunoreactivity (i.e., anti-CD3 antibody, anti-cleaved caspase-3 antibody, TdT enzyme). As an additional negative control, uninvolved tissue sections from diseased patients (when available) were evaluated in conjunction with their respective involved tissue sections. All such controls were appropriately negative for viral antigen, activated caspase-3, and TUNEL.

Results

Apoptosis in HSE in association with productive viral infection. We evaluated 7 patients with acute HSE. Six (86%) of 7 had clear evidence of neuronal and glial apoptosis, as demonstrated by TUNEL and/or activated caspase-3 staining (table 2), with a high degree of congruity noted using both apoptosis detection methods. Apoptotic cells were unequivocally identified as neurons or glia by morphologic characteristics. Neurons made up a larger proportion of apoptotic cells, compared with glial cells. Apoptotic cells did not exhibit late morphologic features of apoptosis (nuclear condensation, margination, or pyknosis), suggesting apoptosis was detected at a relatively early stage at the time that tissues were obtained. Apoptotic cells were restricted predominantly to areas of productive viral infection and did not occur as a widespread, nonspecific process in histologically or virologically uninvolved areas within the same tissue section. Apoptotic neurons and glia were detected in acutely infected patients in regions of disrupted CNS tissue bearing HSV antigen, as measured by TUNEL (figure 1A–C) and/or activated caspase-3 (table 2). In contrast, evaluation of separate areas of anoxic tissue damage in these same patients showed no evidence of productive HSV infection or apoptotic damage, as measured by TUNEL (figure 1D–F) and active caspase-3 staining.

We also evaluated 3 patients with well-documented HSE infection occurring 1–12 years before biopsy or autopsy with chronic neurologic deficits, including dementia, aggressive behavior, language and memory deficits, gait disturbances, and seizure disorder with hemiparesis. Although all patients had histologically abnormal areas of CNS tissue evident by HE stain, none had evidence of ongoing productive viral infection with HSV (as demonstrated by negative HSV specific immunostain). In contrast to acutely infected patients, none of these 3 patients had significant apoptosis, as evidenced by TUNEL (figure 1G–I) or activated caspase-3 staining (table 2).

Apoptosis in congenital CMV CNS disease in association with productive viral infection. We analyzed tissues from a patient

<p>| Table 2. Results summary of patients with herpes simplex virus (HSV) and cytomegalovirus (CMV) central nervous system infection. |</p>
<table>
<thead>
<tr>
<th>Virus, disease stage, patient</th>
<th>HE</th>
<th>Viral immunostain</th>
<th>CD3</th>
<th>Active caspase-3</th>
<th>TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1  Widespread cortical destruction</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>2  Cortical inflammation</td>
<td>+</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>3  Cortical microglious, inflammation</td>
<td>+</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>4  Massive cortical destruction</td>
<td>+ + +</td>
<td>+ +</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>5  Meninges inflamed; diffuse cortical neuronal death, edema, and necrosis beyond number of viral-infected cells; some Cowdry inclusions present</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Artifac (cortex)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Artifac (meninges)</td>
<td></td>
</tr>
<tr>
<td>6  Cortical destruction, no inflammation</td>
<td>+ +</td>
<td>–</td>
<td>+ +</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>7  Cortical microglious, inflammation</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Remote</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8  Severe neuronal loss, multicystic cavitation, lymphocytes, microgliosis</td>
<td>–</td>
<td>–</td>
<td>+ +</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>9  Severe neuronal loss, reactive astrocytosis, dramatic perivascular lymphocytic infiltrates</td>
<td>–</td>
<td>–</td>
<td>+ +</td>
<td>–</td>
<td></td>
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<tr>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>10 Extensive neuronal necrosis and reactive astrocytosis</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Artifact (uninterpretable)</td>
</tr>
<tr>
<td>CMV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 Periventricular and intraparenchymal calcification; many multinucleated giant cells with Cowdry A inclusion bodies; focal micropolygyria of right hippocampus</td>
<td>+ +</td>
<td>–</td>
<td>+ +</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>Subacute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 Marked micropolygyria. Punctate necrosis with calcification throughout white matter; ependymitis; only rare CMV inclusions on multiple sections (except 2 cells)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Remote</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 Polymicrogyria, ependymal destruction; no CMV inclusions</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Artifact (uninterpretable)</td>
</tr>
</tbody>
</table>

NOTE. Scoring indicates relative quantitation of number of positively stained cells in histologically involved areas, ranging from no staining (−) to highest degree of staining (+ + +). HE, Hematoxylin-eosin stain.
Figure 1. Apoptosis in acute herpes simplex virus (HSV) encephalitis (HSE). Sections of central nervous system (CNS) tissue from patient 1 with acute HSE (A–F) and patient 8 with remote HSE (G–I) are shown. CNS tissue from patient 1 with acute HSE demonstrates widespread cortical injury (hematoxylin-eosin stain [HE]; A), including many HSV-productively infected neurons (brown staining, arrowheads; B), and widespread neuronal/glial apoptosis (TUNEL; brown staining, arrowheads; C). In contrast, areas of adjacent cortical injury due to anoxia (D), rather than productive viral infection (E), have no evidence of apoptotic injury (F). CNS tissue from patient 8 with chronic neurologic deficits resulting from HSE 12 years prior to death (remote) shows widespread cortical destruction (G), without evidence of productive viral infection (H), and no evidence of ongoing apoptosis (I). Bar, 20 μm.

with recently-acquired congenital CMV disease and classic periventricular involvement. This patient had widespread Cowdry A inclusion bodies and multinucleated giant cells evident in the periventricular region on HE stain (figure 2A), in conjunction with large numbers of productively infected neurons (as measured by CMV-specific immunostain) in this region (figure 2B). There was nearly a 1:1 correlation between number of CMV-staining cells and apoptotic cells, as evidenced by both TUNEL (figure 2C) and activated caspase-3 techniques (table 2). Areas immediately adjacent to this periventricular concentrated distribution of CMV, as well as an area of focal polymicrogyria in the right hippocampus of this patient, were also evaluated and showed no evidence of histological, virologic, or apoptotic disruption.

We also evaluated 2 patients with severe neurologic sequelae of congenital CMV CNS infection, including marked microgyria, ependymitis, and diffuse white matter punctate necrosis/calcification. These sequelae were the result of infection occurring as recently as 6–8 weeks (subacute) but as long as 3 years (remote) after acquisition of CMV virus in utero. In contrast to the acutely-infected patient described above, we demonstrated minimal evidence of ongoing productive CMV in-
Apoptosis in congenital cytomegalovirus (CMV) central nervous system (CNS) infection. Sections of CNS tissue from patient 11 with acute periventricular CNS disease due to congenitally acquired CMV (A–C), as well as patient 12 with subacute (6–8 weeks prior to analysis) congenitally acquired CMV (D–F). CNS tissue from the acutely infected infant shows many multinucleated giant cells (A), including cells with Cowdry A inclusion bodies (arrows), productive CMV infection (dark brown staining, arrows; B), and evidence of apoptosis (TUNEL, dark brown staining, arrows; C). CNS tissue from the infant with subacute infection showed no periventricular multinucleated giant cells, productive viral infection or apoptosis. In an area of cortical polymicrogyria (hematoxylin-eosin stain [HE]; D) there was only one cell identified with productive CMV infection (E) and no evidence of apoptosis (TUNEL; F). Pale-brown staining seen is nonspecific and not different from that seen in uninfected control cases (data not shown). Bar, 20 μm.

Infection, as evidenced by the absence of CMV inclusions bodies on HE stain, as well as rare (subacute) or absent (remote) CMV-specific immunostained cells in these patients. Neither case patient had significant apoptosis by either activated caspase-3 or TUNEL staining techniques (table 2 and figure 2D–F [subacute]). These results indicate that neuronal/glial apoptosis is associated with productive CMV viral infection acquired in utero and does not persist at timepoints remote from infection, even in patients with severe neurologic sequelae and gyral anomalies occurring weeks to years after acquisition of virus.

Apoptosis in HSV-infected neurons/glia. Having demonstrated apoptosis in CNS tissue of acute HSV-infected patients, we wished to further characterize whether apoptosis occurs in virus-infected neurons/glia or uninfected bystander cells. The majority of cells exhibiting evidence of apoptosis by activated caspase-3 staining were costained for HSV antigen, and these double-stained cells had morphological features identifying them as neuronal or glial in origin (figure 3A). Not all HSV-positive cells were apoptotic. Apoptotic cells were not seen outside areas of viral infection; however, not all apoptotic cells were antigen positive, suggesting that apoptosis occurs both as a direct consequence of viral infection and also through indirect bystander mechanisms in cells with close proximity to infected cells.

Apoptosis in CMV-infected neurons. Having demonstrated apoptosis in CNS tissue of a patient with acute CMV infection, we wished to further characterize whether apoptosis occurs in virus-infected neurons/glia or uninfected bystander cells. A large proportion of cells exhibiting evidence of apoptosis by activated caspase-3 staining were costained for CMV antigen, and these double-stained cells had morphological features unequivocally identifying them as of neuronal origin (figure 3B), often occurring in multinucleated giant cells bearing Cowdry A inclusions. In contrast to HSV infection (in which the number of virus-infected cells was substantially greater than apoptotic cells), the number of CMV-infected cells closely matched the number of apoptotic cells. This suggests that, in distinction to HSV infection, in which both direct and bystander apoptosis occur, in CMV infection, apoptosis results predominantly from direct viral injury to infected cells.

Neuronal apoptosis not dependent on inflammatory T cell responses to HSV or CMV infection. We wished to determine whether apoptosis resulted as a consequence of viral infection
Figure 3. Costaining for apoptosis and productive viral infection. Sections of central nervous system (CNS) tissues from patient 1 with acute herpes simplex encephalitis (HSE; A) and patient 11 with acute cytomegalovirus (CMV) CNS infection (B) were costained for evidence of productive viral infection (HSV or CMV-specific antibody, indicated by red staining), as well as apoptosis (activated caspase-3 antibody, indicated by brown staining). In both acute HSV and acute CMV CNS infections, multiple neurons and glial cells were identified which were both infected with virus and undergoing apoptosis (colabeled cells are indicated by maroon staining, large arrows). Examples of cells with productive viral infection, but not undergoing apoptosis (white arrowheads), as well as cells undergoing apoptosis without productive viral infection (black arrowheads) were also present in both CMV and HSV infection. Bar, 20 μm.

independent of the host immune response or alternatively, resulted from immune-mediated killing of virus-infected cells. We, therefore, examined the association between the degree of inflammatory response and the presence of apoptosis. Neuronal and glial apoptosis were noted in acutely infected patients, regardless of the degree of inflammatory T cell infiltrate (as measured by activated caspase-3 and CD3-labeling). The majority of acutely infected patients had significant inflammation as a component of their disease, but CD3-positive labeled cells represented a distinct population from apoptotic cells (as evidenced by lack of colabeling with activated caspase-3) (figure 4A). As a corollary observation, we still detected high levels of neuronal/glial apoptosis in an acutely infected patient who did not mount any inflammatory response to viral infection (table 2). Furthermore, despite the presence of extensive inflammatory cell infiltrates in 2 of 3 remotely infected patients (as evidenced by large numbers of CD3-positive staining cells), inflammatory T cells were not found to be apoptotic (no activated caspase-3 costaining) nor were adjacent neurons/glia (figure 4C). These results suggest that the inflammatory response and the apoptotic program are distinct (although possibly interacting) processes in HSV-infected patients.

CMV-infected infants had less T cell inflammatory response to infection, compared with HSV-infected patients, consistent with the fact that infants have relatively immature immune responses, compared with older children and adults. In spite of this less-robust inflammatory response, abundant neuronal/glial cell apoptosis was noted in the acute CMV-infected infant, as described earlier (figure 2). In areas where CD3-positive inflammatory cell infiltrates were detected, T cells represented a distinct population from cells exhibiting evidence of apoptosis, as measured by activated caspase-3 (figure 4D).

Taken together, results from the evaluation of tissues from all HSV and CMV-infected patients indicate that apoptosis occurs in neurons/glia of acutely infected patients, in close temporal and spatial association with productive viral infection, and is not dependent on secondary inflammatory T cell responses. Absence of apoptosis in age-matched control subjects without CNS infection. To assess the degree of baseline apoptosis in CNS tissues as a function of age, we analyzed autopsy tissues (hippocampal sections) from uninfected patients who died from other causes. Tissue samples from 11 patients representing each decade of life from infancy to late adulthood were analyzed and showed minimal (infancy) or no (all other age groups) evidence of apoptosis, as measured by activated caspase-3 staining (data not shown). These results lend further support to the finding that the apoptosis that occurs in neurons/glia of HSV and CMV-infected patients is a pathologic process, distinct from normal developmental or age-related changes.

Discussion

Our study is the first demonstration of apoptotic neuronal death in patients with acute viral CNS infection, specifically in patients with HSE and congenital CMV infection. In addition to demonstrating neuronal/glial apoptosis in regions of the brain with acute productive viral infection, we demonstrate that apoptosis is not found in brains of patients with severe CNS injury and neurologic symptoms months to years after acute viral infection. In both CMV and HSV infection, the presence and degree of apoptosis did not correlate with the presence or magnitude of inflammatory response, indicating that apoptosis is a direct consequence of viral injury, rather than a secondary effect of virus-induced inflammatory responses. Our findings imply that apoptotic neuronal death is an important component
of CNS injury and destruction in the acute phase of infection of patients with viral CNS infection.

The correlation between the number of infected neurons and the degree of apoptotic neuronal death differed in HSV and congenital CMV infection. Although only one case of acute congenital CMV infection was available for analysis, CMV infection was associated with a nearly direct (1:1) correlation between the number of infected neurons and the number of apoptotic neurons, whereas tissues from patients with acute HSV infection showed many more infected nonapoptotic neurons than infected apoptotic neurons. There are several potential explanations for this difference in degree of observed apoptosis. First, HSV and CMV may differ in their capacity to induce apoptotic-signaling pathways in neuronal cells. Strain-specific differences in the capacity to induce apoptosis have been described in other viral models of neuronal apoptosis [74], and this may apply to different members of the herpesvirus family, as well. Alternatively, although we chose to analyze only immunocompetent hosts in this study, congenitally infected CMV patients are, to some degree, less immunocompetent at the time of their productive viral infection than older children and adults at the time of their acute HSV infections. Therefore, CMV-infected infants could be prone to higher degrees of direct virus-mediated apoptotic injury at the time of acute infection. Finally, the observed difference may be related to the relative tempo by which infection proceeds in HSV, compared with CMV-infected patients. The clinical presentation and histopathology associated with these 2 diseases are markedly different. In HSV infection, neurons/glia are rapidly infected, resulting in an overwhelming, lytic, hemorrhagic destructive process. It is rare to observe viral inclusion bodies and/or multinucleated giant cells in CNS tissues of patients with HSV infection. In contrast, neurons infected with CMV typically accumulate large amounts of virus prior to destruction, as reflected by easily observable Cowdry A viral inclusions within infected multinucleated giant cells. One potential consequence of this difference is that identification of infected cells on the basis of prominent cytomegaly in the case of CMV infection may greatly facilitate identification of infection and apoptosis in identical cells using sequential sections. Additionally, the accumulation of CNS injury and destruction in the acute phase of infection of patients with viral CNS infection.

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of large amounts of virus within an infected cell prior to cell lysis (as occurs in CMV) may in some manner potentiate the initiation of the apoptotic program.

Our study indicates that, in HSV and CMV infection, CNS apoptosis appears to result as a direct consequence of viral infection of neurons, rather than as a secondary consequence of virus-induced inflammatory T cell responses or in infiltrating T cells themselves. This is in contrast to experimental Murray Valley Encephalitis infection, in which inflammatory infiltrate includes apoptotic macrophages and lymphocytes [10]. Similarly, during experimental Semliki Forest Virus encephalitis, infiltrating leukocytes and neural precursor cells undergo apoptosis while productively infected neurons undergo necrosis [27]. However, apoptotic inflammatory cells may also secrete factors that induce apoptosis in neighboring cells, as seen in the case of HIV infection, in which infiltrating mononuclear phagocytes secrete a variety of factors that mediate neuronal apoptosis [75, 76]. Although apoptosis appears to be a direct effect of viral infection, rather than an indirect effect of proinflammatory cytokines, in HSE and congenital CMV CNS infection, it is possible that inflammatory responses provide an additional mechanism of tissue injury, and could interact in some way with virus-induced apoptotic signaling processes.

There are many plausible reasons why neuronal apoptosis likely has biological significance in viral infections of the human CNS. A complex network of intracellular signaling cascades is activated by apoptosis-inducing stimuli in neurons. In addition to the basic apoptosis-related biochemical and morphological changes seen in neuronal cells, apoptotic neurons also undergo alterations in neurotransmitter release, growth factor secretion, and cell-cell interactions. These changes can have deleterious effects on neighboring neurons during the primary insult, often resulting in a widening of the area of neuronal loss at timepoints directly following the initial insult [77].

This study suggests that apoptosis is an important mechanism of virus-induced neuronal and glial cell death in the CNS of humans with viral encephalitis. These results have importance not only for an improved understanding of the pathogenesis of human encephalitis but also for the potential development of novel therapeutic strategies. The importance of apoptotic death in virus-induced CNS disease has been underrecognized, due in part to the fact that classic histopathologic descriptions of encephalitis were made prior to the distinction between apoptotic and necrotic cellular death. We have demonstrated elsewhere the protective effect of apoptotic blockade in a murine model of viral myocarditis [78], and this study indicates that similar intervention may be may be useful as an adjunct to the treatment of patients with acute, but not remote, viral CNS infection. Identification of specific mechanisms underlying virus-induced injury in human viral encephalitis, including apoptosis, may lead to improved, novel and/or adjunctive strategies for the treatment of this devastating disease.

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References