SHORT COMMUNICATION

Apoptosis in Acute and Chronic Central Nervous System Disease Induced by Theiler's Murine Encephalomyelitis Virus

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Apoptosis has been observed in neural development and in various neurological diseases, including viral infection and multiple sclerosis. Theiler's murine encephalomyelitis virus is divided into two subgroups based on neurovirulence: the highly neurovirulent GDVII strain produces an acute fatal polioencephalomyelitis in mice, whereas the attenuated DA strain produces demyelination with virus persistence preceded by an acute infection. TUNEL combined with immunocytochemistry was used to detect apoptosis in the central nervous system and to characterize which cell types were involved during the acute stage in both GDVII and DA virus infection and during the chronic stage in DA virus infection. We found that during the acute stage, apoptosis was induced in neurons in both virus infections. However, the number of apoptotic neurons was much greater in GDVII virus-infected mice than in DA virus-infected mice (P < 0.01). During the chronic stage of DA virus infection, apoptotic cells were detected only in the spinal cord white matter. Some of these cells were dual labeled for fragmented DNA and carbonic anhydrase II, an oligodendrocyte marker. Our results indicate that apoptosis of neurons could be responsible for the fatal outcome in GDVII virus infection. In contrast, apoptosis of oligodendrocytes can contribute to the chronic demyelinating DA virus infection.

Apoptosis, or programmed cell death, has recently been observed in neural development and in a wide variety of neurological diseases including viral infection (1, 2). For example, Sindbis virus causes a fatal encephalomyelitis in neonatal mice, but replicates in the adult mouse brain without producing significant cytopathology. Griffin et al. demonstrated that increased Sindbis virus neurovirulence was associated with apoptosis (3, 4). The involvement of apoptosis was also demonstrated in chronic persistent neurological virus infections, such as HIV encephalitis (5) and HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (6) and its experimental animal model, HAM rat disease (7). Recently, oligodendrocyte and mononuclear cell apoptosis in the central nervous system (CNS) has been demonstrated in multiple sclerosis (MS) (8, 9) and its experimental model, experimental allergic encephalomyelitis (EAE) (10–12).

Theiler's murine encephalomyelitis viruses (TMEV) belong to Picomaviridae and are divided into two subgroups based upon their neurovirulence in mice, i.e., GDVII and TO. The GDVII strain, a highly neurovirulent strain, causes an acute fatal polioencephalomyelitis in mice (13, 14), whereas the disease induced by the DA strain (TO subgroup) is biphasic (15). An early disease of polioencephalomyelitis develops several days after DA virus infection. In contrast to GDVII strain, the mice survive the acute stage and progress to develop a chronic demyelinating disease with viral persistence in the spinal cord. This chronic stage is a well-characterized experimental model for MS (16, 17).

We studied the acute and chronic CNS disease induced by the GDVII and DA strains of TMEV by combining immunocytochemistry with terminal deoxynucleotidyl transferase-mediated dUTP–biotin nick-end labeling (TUNEL) (18). The objectives were twofold: first, to ascertain whether the GDVII versus DA strain differences in the neurovirulence were associated with apoptosis and second, to characterize apoptosis in the chronic demyelinating CNS lesion in DA virus-infected mice.

After GDVII virus infection all infected mice died or became moribund and were sacrificed within 2 weeks. TUNEL-positive cells were readily detected in the brain parenchyma (5337 ± 888 cells/brain section) at 1 week p.i. and were primarily neurons (Fig. 1A). In addition, 5.7 ± 1.2% of the infiltrating perivascular mononuclear cells...
were TUNEL positive. The TUNEL-positive cells had the characteristic appearance of apoptosis (Fig. 1A, inset). Some exhibited chromatin condensation around the margin of the nucleus, forming either crescent caps or rings, while other nuclei appeared dense brown with rounded or oval apoptotic bodies. Groups of TUNEL-positive neurons were widely distributed in the cerebral cortex, the hippocampus, and the olfactory nucleus. In the spinal cord, TUNEL-positive cells were frequently found in the center of neuronophagia in gray matter, although these cells were present in both gray and white matter. Occasional white matter cells in the spinal cord were dual labeled for both fragmented DNA and carbonic anhydride II (CA II) (19) (data not shown).

Viral-antigen-positive cells (4866 ± 867 cells/brain section) (20) were widely scattered throughout the brain and spinal cord and their distribution was similar to that of the TUNEL-positive cells (Fig. 1C). Viral antigen was observed only in the cytoplasm (Fig. 1C, inset). Although many apoptotic cells could be identified as TMEV-infected cells by combined TUNEL/immunohistochemistry for viral antigen, some completely TMEV-negative cells were TUNEL positive and some TMEV-positive cells were TUNEL negative (Fig. 1G).

While the inflammatory cell response during GDVII virus infection was subtle (Fig. 1E), TUNEL- and viral-antigen-positive cells were seen throughout the brain and spinal cord. A mild perivascular cell infiltration was sporadically observed in both the gray and the white matter. However, in the parenchyma, RCA-1-positive microglia or macrophages (21) were frequently present (Fig. 1H) but negative for viral antigen (22). It was also noted that the mononuclear cell infiltration was usually more intense in the areas with better preservation of the tissue architecture versus areas in which viral-antigen-positive apoptotic cells were frequently present. Interestingly, neurons in these infamed areas where relatively well preserved and their nuclei were negative for apoptosis by the TUNEL assay (data not shown). The paucity of inflammatory cells and activation of microglia in the areas, in which apoptotic cells were present, was consistent with the general observations that apoptosis per se does not induce an inflammatory response (23) and phagocytosis of the apoptotic cells by tissue macrophages is a characteristic feature of apoptosis (24).

All mice were clinically asymptomatic during the first 4 weeks of observation after DA virus infection. At 1 week p.i. the distribution of TUNEL and/or viral antigens for DA virus infection was similar to that in GDVII virus-infected mice. However, the numbers of apoptotic neurons (703 ± 135 cells/brain section) and viral-antigen-positive cells (216 ± 71 cells/brain section) were much smaller (P < 0.01, Mann–Whitney U test) in DA virus infection versus GDVII virus infection (Figs. 1B and 1D). A few small clusters of TUNEL-positive neurons were found in the cerebral cortex, the hippocampus, and the olfactory nucleus. TUNEL-positive mononuclear cells were also encountered among the perivascular cell infiltration (3.9 ± 0.5%). In the spinal cord, TUNEL-positive cells were present in both the gray and the white matter, and some of these cells were oligodendrocytes based on their size and localization (Fig. 2A) and CA II labeling (data not shown).

In contrast to acute fatal disease in GDVII virus infection, the perivascular infiltration in DA virus infection was conspicuous (Fig. 1F). In inflammatory lesions, neurons were relatively well preserved and the number of large neurons appeared not to be reduced.

In the chronic stage of disease, a month or more after infection with DA virus, mice developed an ataxic hind-limb paralysis. The inflammation in the brain and spinal cord gray matter had largely subsided, and TUNEL and viral-antigen-positive cells were seldom observed. Instead, TUNEL-positive cells were scattered throughout the demyelinated spinal cord white matter. The location of some of these cells in the white matter and their size suggested that they were oligodendrocytes. Combined TUNEL/immunohistochemistry demonstrated that 5% TUNEL-positive cells were immunoreactive for CA II, thus identifying them as oligodendrocytes (Fig. 2B), while others were immunoreactive for RCA-1 (Fig. 2C). TUNEL-positive nuclei were not detected in GFAP-positive astrocytes (Fig. 2D). Although TUNEL-positive cells were always seen in association with viral-antigen-positive cells, TUNEL-positive nuclei never colabeled with viral antigen (Fig. 2E). TUNEL-positive cells were virtually absent in perivascular infiltrating cells in the spinal white matter (0.4 ± 0.2%). To further characterize the apoptosis during the chronic demyelinating stage of TMEV infection, we compared the cell type of apoptotic cells in TMEV infection and acute EAE immunized with an encephalitogenic
myelin proteolipid peptide (P3) in adjuvant as described previously (25). In paralyzed EAE mice, occasional white matter cells and 8.0 ± 2.3% of perivascular infiltrating cells were TUNEL positive.

To determine whether TMEV infection per se induced apoptosis of neurons without infiltrating mononuclear cells or activated glial cells, mouse neuroblastoma cells (Neuro-2a) (26) were infected with GDVII virus or DA virus in vitro. TMEV infection was confirmed by immunofluorescent staining for viral antigens and a plaque assay using BHK-21 cells (data not shown). At 12 hr p.i., the TUNEL staining revealed a distinct population of the cells that were labeled for DNA fragmentation in both GDVII and DA virus infection and had a characteristic appearance of apoptosis (Fig. 3A). No TUNEL-positive nuclei were seen in mock-infected culture (Fig. 3B).

The present study clearly demonstrated that in the acute stage of TMEV infection, apoptosis was induced in CNS neurons in both GDVII and DA virus-infected mice. The number of apoptotic neurons, however, was much greater in GDVII virus infection than with DA virus infection. This apparent difference in the number of apoptotic neurons between GDVII versus DA virus infection contributes to the TMEV subgroup-specific neurovirusence.

Induction of apoptosis in TMEV-infected neuroblastoma cells in vitro suggested that TMEV infection could induce apoptosis in the absence of infiltrating mononuclear cells or activated glial cells in vivo. Combined TUNEL/immunohistochemistry for viral antigens showed many of the TUNEL-positive neurons in CNS were also infected with TMEV.

The reduced sensitivity to virus-induced apoptosis or survival of neurons in DA virus infection may be ascribed to apoptosis-inhibiting molecules, such as cytokines, produced by infiltrating mononuclear cells and/or activated glial cells. However, this does not exclude the possibility that a viral gene product(s) from the DA virus is involved in preventing apoptosis as observed in other viral systems (27, 28). Tolskaya et al. (29) showed that poliovirus, a representative of Picornaviridae, encodes products which trigger and prevent the development of apoptosis in infected cells. Whatever the mechanism of its inhibition, blocking premature death by apoptosis of the host cell should provide significant advantages for persistence of DA virus.

Why should there be a suicide program triggered by virus infection built into the neuron? In case of neuronal virus infection, elimination of infected neurons by virus-specific T cells would not be expected since neurons are incapable of expressing, or express very few, major histocompatibility complex molecules (30). We hypothesize that apoptosis of infected neuronal cells may be a protective mechanism against CNS viral infection in the absence of humoral and cellular immune responses or prior to the generation of an immune response. Elimination of virus-infected host cells by apoptosis prior to the assembly of infectious virus could inhibit viral replication in the CNS which is proposed as a mechanism for inactivation of virus by cytotoxic T cells (31, 32). Alternatively, activation of endogenous, nonlysosomal endonuclease, a very early event in apoptosis, might destroy or disable emerging virus (31). Viewed in this light, in the GDVII virus-infected host, elimination of the viral-infected neurons by the induction of apoptosis may be a result of a defensive mechanism against viral infection, although the outcome is death of the animal.

During the chronic stage of DA virus infection, apoptotic nuclei were never colabeled with viral antigen. Thus, the mechanism of apoptosis in oligodendrocytes is likely to be different from that for neurons observed in the acute stage of disease. Lipton et al. (33) found that the predominant virus antigen burden resides within macrophages and suggested that TMEV-infected macrophages play an important role in the development of chronic white matter lesions in DA virus infection. In support of this view, TUNEL-positive cells were always seen contiguous to the TMEV-infected cells in the present study. Although the implication of this finding needs to be clarified in future experiments, undefined factors derived from TMEV-infected macrophages and/or "by-stander" macrophages recruited and activated by TMEV-specific T cell (34), such as nitric oxide, cytokines, and chemokines, may play a crucial role in the induction of oligodendrocyte apoptosis.

In this report, we demonstrate for the first time that apoptosis of neurons and oligodendrocytes is associated with the fatal outcome in acute disease and demyelination in chronic disease of TMEV infection, respectively. The apoptosis of neurons, oligodendrocytes, and mononuclear cells could be fundamental to other CNS diseases, such as acute viral encephalitis, MS, and HIV.
encephalitis, and the elucidation of the mechanism of apoptosis appears to be a key for a better understanding of these CNS diseases.

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