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Is the Cell Death in Mesial Temporal Sclerosis Apoptotic?

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Summary: *Purpose:* Mesial temporal sclerosis (MTS) is characterized by neuronal loss in the hippocampus. Studies on experimental models and patients with intractable epilepsy suggest that apoptosis may be involved in neuronal death induced by recurrent seizures.

Methods: We searched evidence for apoptotic cell death in temporal lobes resected from drug-resistant epilepsy patients with MTS by using the terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP (TUNEL) method and immunohistochemistry for Bcl-2, Bax, and caspase-cleaved actin fragment, fractin. The temporal lobe specimens were obtained from 15 patients (six women and nine men; mean age, 29 ± 8 years).

Results: Unlike that in normal adult brain, we observed Bcl-2 immunoreactivity in some of the remaining neurons dispersed throughout the hippocampus proper as well as in most of the reactive astroglia. Bax immunopositivity was increased in almost all neurons. Fractin immunostaining, an indicator of caspase activity, was detected in ∼10% of these neurons. Despite increased Bax expression and activation of caspases, we could not find evidence for DNA fragmentation by TUNEL staining. We also could not detect typical apoptotic changes in nuclear morphology by Hoechst-33258 or hematoxylin counterstaining.

Conclusions: These data suggest that either apoptosis is not involved in cell loss in MTS, or a very slow rate of cell demise may have precluded detecting TUNEL-positive neurons dying through apoptosis. Increased Bax expression and activation of caspases support the latter possibility. **Key Words:** Epilepsy—Cell death—Bcl-2—Bax—Caspases.

The most prominent histopathologic finding in drug-refractory temporal lobe epilepsy associated with mesial temporal sclerosis (MTS) is selective neuronal loss of varying severity in the hippocampus (1,2). The mechanism of neuronal death in MTS is unknown. Recent evidence from experimental studies suggests that apoptosis may be involved in neuronal death after recurrent seizures. Several markers of apoptotic cell death have been detected in rodent models of epilepsy induced by kainic acid, pilocarpine or repetitive perforant pathway stimulation (3–7). Moreover, demonstration of nuclear expression of the cell-cycle protein, cyclin B, associated with increased Bax expression in hippocampal neurons of patients with MTS, suggests apoptosis as a way of cell death in this region (8). Recently, alterations in Bcl-2 family proteins and cleavage of pro-caspases 1 and 3 have been reported in human temporal lobe specimens resected from patients with intractable epilepsy (9). In this study, the presence of active forms of caspase-1 and caspase-3 in epileptic tissue samples was considered evidence for programmed cell death, even though DNA fragmentation was detected by the terminal transferase–mediated d-UTP nick-end labeling (TUNEL) technique in only two of the six brains examined (9).

Apoptosis, a form of programmed cell death, was first identified during embryogenesis (10). During postnatal life, apoptosis plays a role in controlling the number of cells (e.g., in intestinal villi) and in involution of some tissues (e.g., thymus, pineal gland). Apoptosis also can mediate cell death during acute (e.g., ischemia) and slowly progressing neurologic diseases (e.g., Alzheimer disease) (11–14). Internucleosomal DNA fragmentation is considered to be a biochemical hallmark of apoptosis and can be detected by the TUNEL staining of brain specimens (15,16). This simple method has been very useful in identifying apoptotic cell death in several CNS disorders (11–14,17). However, the initial enthusiasm for detecting TUNEL-positive cells as a marker of apoptosis has recently been replaced by more cautious approaches because TUNEL positivity also was observed in necrotic cells (16,18). Research over the past years has repeatedly demonstrated that an unequivocal demonstration of apoptosis in pathologic cell death usually requires concurrent detection of several biochemical markers of the apoptotic

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cascade in addition to the histologic criteria and TUNEL staining (16). An increase in the Bax/Bcl-2 ratio (19–21), demonstration of cleaved active forms of caspases (22,23), and detection of the caspase-cleavage products (17) have been used to disclose the apoptotic cascade in several neurologic diseases. Recently, the caspase-cleaved actin fragment, fractin, also has been proposed as a valuable marker of apoptosis in various tissues including brain (24–26). Supporting this view, fractin immunopositivity has been reported to be correlated with TUNEL staining in the ischemic brain (27,28).

Although detection of alterations in the Bcl-2 family proteins and of the cleaved active form of caspases suggests activation of the apoptotic cascade in intractable epilepsy, TUNEL staining could be demonstrated in only a small number of brain specimens from patients with MTS (9). Increasing evidence indicates that several markers of apoptosis can be detected in dying cells with nonclassic forms of apoptosis as well (29,30). Because the histologic appearance of affected neurons is not quite typical for apoptosis, we reevaluated the involvement of apoptosis in MTS by using the TUNEL method, Bcl-2, Bax, and fractin immunohistochemistry. All of these markers could be identified by antibodies that work on paraffin-embedded tissues and therefore were suitable for examining the previously collected patient material. Nuclear morphology of the affected cells also was examined by Hoechst-33258 and hematoxylin counterstaining to disclose apoptotic morphologic changes.

SUBJECTS AND METHODS

This study was performed on temporal lobe specimens obtained from 15 patients, six women and nine men (mean age, 29 ± 8 years), who underwent surgery for drug-refractory mesial temporal lobe epilepsy in the Epilepsy Units of Sevgi Hospital and Hacettepe University Hospitals (Ankara, Turkey). Clinical variables of the patients are shown in Table 1. Only the specimens that were histopathologically diagnosed as hippocampal sclerosis were selected; specimens with other or additional pathologies were not included. Control hippocampal sections, which displayed no macro- or microscopic abnormalities, were obtained from three adults (aged 48 to 60 years), who died of a traffic accident (due to peripheral bleeding), acute myocardial infarction, and an unidentified cause.

Patients selected for surgery had failed to respond to optimal medical management for a minimum of 2 years before surgery and had localizing interictal spikes. All patients had cranial magnetic resonance imaging (MRI) before and after surgery. Unilateral atrophy (diagnosed based on measurement of bilateral hippocampal volumes) and hippocampal hyperintensity were used as the diagnostic markers of hippocampal sclerosis. Ictal and interictal single-photon emission computed tomography (SPECT) was performed if necessary. Ictal and interictal EEG recordings were obtained in all patients with long-term video-EEG monitoring from the scalp. Subdural stripes and grids were used in some of the patients to localize the focus precisely. Standard neuropsychological tests in all and the Wada test in six patients were performed before surgery. Selective amygdalohippocampectomy, anteromedial temporal lobectomy, or standard anterior temporal lobectomy with amygdalohippocampectomy was used for resection of the epileptic focus.

TABLE 1. *Summary of clinical data for patients with mesial temporal sclerosis*

Patients	Age at operation (yr)	Sex	Seizure type	Duration of seizures (yr)	Operation side
1	37	F	CPS, GTCS	17	R
2	27	M	GTCS	10	R
3	23	M	CPS, GTCS	8	L
4	41	M	CPS, GTCS	12	R
5	35	M	CPS, GTCS	13	R
6	24	F	CPS	7	L
7	16	M	CPS, GTCS	6	L
8	26	M	CPS, GTCS	10	R
9	26	M	CPS	20	R
10	25	M	CPS	18	L
11	40	F	CPS, GTCS	29	L
12	24	F	CPS, GTCS	5	L
13	32	M	CPS, GTCS	25	L
14	22	F	CPS, GTCS	17	R
15	44	F	CPS, GTCS	34	R

CPS, Complex partial seizure; GTCS, generalized tonic–clonic seizure; R, right; L, left.

Tissue preparation

En bloc hippocampal resections were fixed in 10% formalin before routine processing in paraffin. Serial coronal sections 5-μm-thick passing through the hippocampal formation were obtained and processed routinely. Sections were stained with hematoxylin and eosin (H&E) and TUNEL, and with antisera against the glial fibrillary acidic protein (GFAP), Bcl-2, Bax, and fractin. All slides were examined by two pathologists (T.E. and F.S.) and two neurologists (T.D. and B.E.) separately and together.

Detection of apoptotic cells with TUNEL method

DNA fragmentation was detected in situ by 3′-OH end labeling with a kit containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP according to the manufacturer's recommendations (ApopTag®; Oncor; Inc., Gaithersburg, MD, U.S.A.). Tissue sections were deparaffinized and dehydrated. After proteinase K incubation for 30 min, endogenous peroxidase was blocked with H_2O_2. Sections were incubated in a buffer containing TdT and digoxigenin-labeled dUTP, followed by digoxigenin-conjugated peroxidase treatment. Diaminobenzidine (DAB) was used as chromogen, and methyl green, for counterstaining. Positive (human medulloblastoma) and negative (TdT omitted) controls were included.

Immunohistochemistry for GFAP, Bcl-2, and Bax proteins

Sections were microwaved in citrate buffer at pH 6.0 for two 5-min cycles for antigenic retrieval and then were immunostained with anti-GFAP (Dako; 1:50 dilution) and anti-Bcl-2 (Boehringer-Manheim; 1:1,000 dilution) antibodies. The conventional avidin-biotin-peroxidase technique was carried out, and DAB was used as a chromogen. Nuclei were visualized by hematoxylin counterstaining. Positive and negative controls were included in each experiment. Control and MTS sections were processed simultaneously to ensure standard staining conditions. The tyramide amplification kit [T-20923, TSA Kit 13 with horseradish peroxidase (HRP) goat anti-rabbit immunoglobulin G (IgG) and Alexa Fluor 546 tyramide; Molecular Probes, Leiden, The Netherlands] was used for immunolabeling with anti-Bax antibody (Dako; 1/1,500 dilution) according to the manufacturer's protocol. The sections were coverslipped with mounting solution containing Hoechst-33258 (H-3569; Molecular Probes).

All procedures were performed at room temperature. Sections were examined under a microscope (Nikon Eclipse E600 with Nikon Y-FL Epi-fluorescence attachment, Tokyo, Japan). The images were captured by a TV lens (C-0.6X) and a digital camera (Nikon DXM 1200) at a resolution of 1,280 × 1,024 pixels. The UV-2A (EX: 330–280, DM: 400, BA: 420) filter was used for viewing Hoechst-33258 fluorescence, and the G-2A (EX: 510–560, DM: 575, BA: 590) filter for Bax fluorescence. Digital images were fed to a computer and processed with ACT-1 (version 2) image-processing software provided by the manufacturer (Nikon).

Detection of caspase-cleaved actin fragment

Immunohistochemistry for the caspase-cleaved actin fragment was detected by the protocol described in our previous reports (27,28). Antigen retrieval was achieved by microwaving in 1 m*M* EDTA buffer (pH, 8). Polyclonal rabbit fractin antibody (generously provided by Drs. F. Yang and G. Cole of UCLA) was used at 1/5,000 dilution in 3% bovine serum albumin for immunolabeling. The conventional avidin-biotin-peroxidase technique was carried out, and DAB was used as chromogen with hematoxylin counterstaining. The same sections also were coverslipped with a mounting medium containing Hoechst-33258. Light microscopic and fluorescence images were obtained from the same sections, which were captured and digitized as indicated earlier and then were superimposed by using Adobe Photoshop 5.0 to study the nuclear morphology in fractin-positive cells.

RESULTS

Subjects

Clinical features and pathologic findings of the patients are summarized in Table 1. Mean duration of the epilepsy was 15.4 ± 9 years. All patients had normal neurologic examinations before surgery. Eight of 15 specimens were resected from the right hemisphere. Most of the patients had four to six seizures per month, whereas one patient had one to three, and another one, eight to 10 seizures per month. Thirteen patients had a seizure within the last 7 days (seven of them; within 72 h). One patient's last seizure was reported to be 10, and another one's was 25 days ago.

Histopathologic findings

The examination of H&E-stained hippocampal sections revealed characteristic findings of MTS in all patients (marked depletion in pyramidal neurons and gliosis in the cornu ammonis fields; CA 1, 3, and 4 with relative sparing of CA2). These pathologic changes ended abruptly at the subiculum. A less-striking neuronal cell loss in the dentate gyrus also was noted in most of the cases.

Immunohistochemical findings

Hippocampal sections adjacent to H&E-stained sections were evaluated for TUNEL positivity and, GFAP, Bcl-2, Bax, and fractin immunoreactivity in comparison to serial sections obtained from the rostrocaudal extend of the hippocampi of similarly fixed and processed normal adult brains. Gliosis, identified by the presence of GFAP-positive reactive astrocytes, was seen in areas of neuronal loss and increased in parallel with the intensity of neuronal loss in all cases (data not shown). In accordance with previous reports (8,31–33), no significant Bcl-2 staining appeared in hippocampal sections from the normal brain, whereas mild to moderate cytoplasmic immunopositivity was detected in some of the residual neurons in hippocampi of the MTS patients (Fig. 1A). These neurons were scattered in all main sectors of the affected hippocampi. In areas of reactive gliosis, numerous astroglia and presumably microglia immunopositive for Bcl-2 were seen (Fig. 1B). A mild to moderate degree of Bax immunoreactivity was detected in control brain sections, mainly in cells with neuronal phenotype by morphologic criteria. In brain sections from MTS patients, most of the residual pyramidal neurons in the CA2 and CA3 sectors, and also the severely affected dentate granule layer showed more intense cytoplasmic immunoreactivity for Bax compared with that in the normal adult hippocampus (Fig. 1C). Hoechst-33258 counterstaining revealed that these cells had a normal nuclear morphology (Fig. 1D).

No fractin immunopositivity was seen in the normal hippocampus, as reported before (26). About 10% of the residual neurons in the hippocampus of patients with MTS showed moderate to intense fractin immunostaining (Fig. 1E). Most of these neurons were shrunken and dispersed throughout the hippocampus, but in some brains, were more numerous in the dentate granule layer and CA2–3 sectors. Only a few glial cells with morphologic

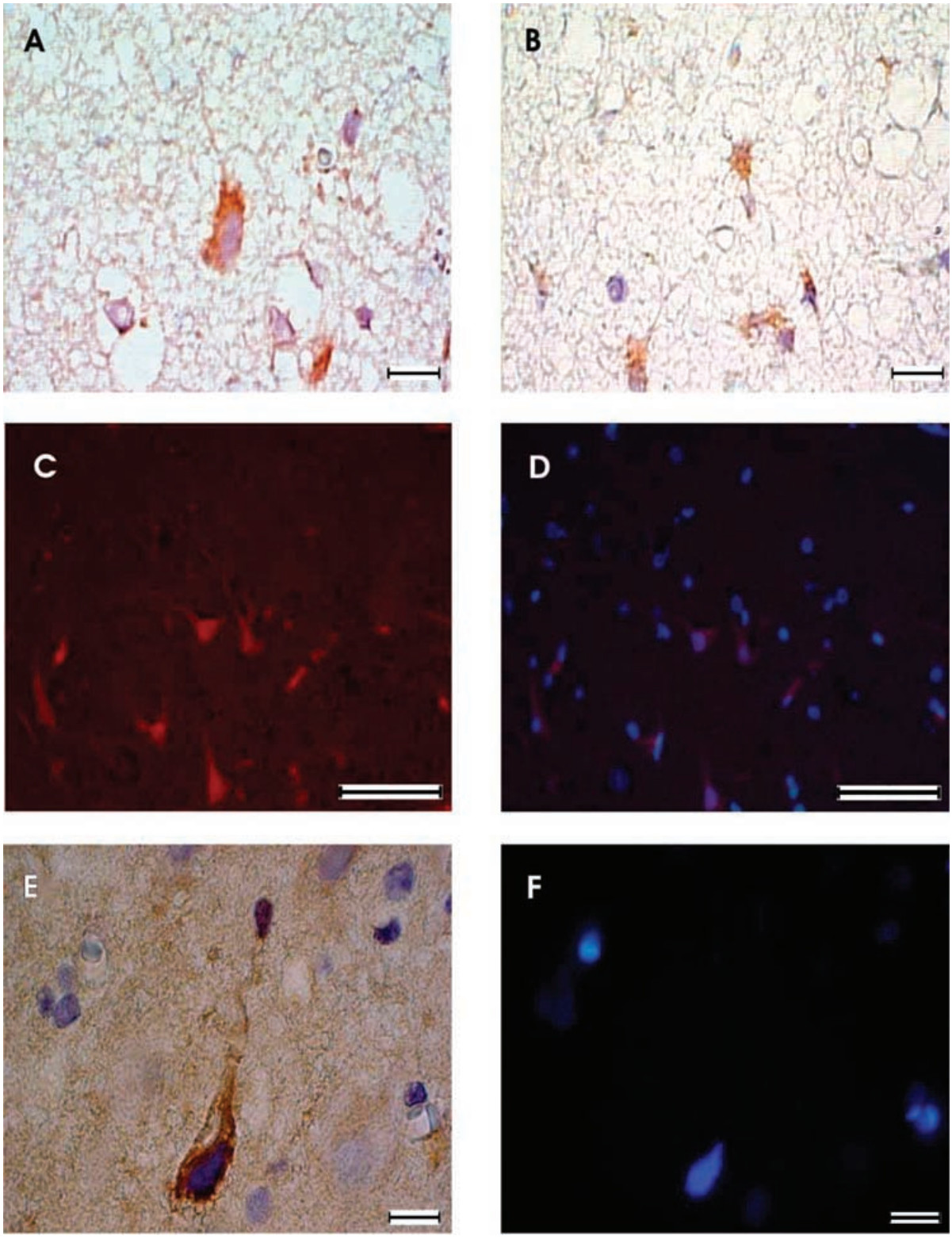


FIG. 1. Residual neurons in the hippocampus of patients with mesial temporal sclerosis (MTS) displayed increased Bcl-2 and Bax expression and caspase activity, suggesting activation of the apoptotic pathways in these cells but no nuclear morphology compatible with apoptosis. **A:** A residual neuron expressing Bcl-2. **B:** Bcl-2 expression in the astroglia and microglia in an area of reactive gliosis. **C:** Most of the pyramidal neurons in the hippocampus of MTS patients showed intense cytoplasmic Bax immunoreactivity. **D:** Hoechst-33258 counterstaining reveals that these cells have a normal nuclear morphology. **E:** Immunostaining for the caspase-cleaved actin fragment, fractin, demonstrates intense immunopositivity in a residual pyramidal neuron. **F:** The nucleus does not display apoptotic features with Hoechst-33258 staining. Scale bar is 20 μm in **A, B, E, F**, and 100 μm in **C** and **D**.

features suggestive of microglia were immunopositive for fractin. Conversely, no TUNEL-positive cells in serial sections of the resected tissue were seen from MTS cases despite strongly positive cells in medulloblastoma sections used as positive control and processed simultaneously. TUNEL staining was repeated 4 times on different days in all cases, and any positive nuclei were rigorously searched by four investigators, as indicated in the Methods. In parallel with the TUNEL data, we could not detect any nuclei having typical apoptotic morphology with Hoechst-33258 or H&E staining. Fractin-positive cells also did not display apoptotic nuclear features when examined by Hoechst-33258 or hematoxylin counterstaining (Fig. 1F).

DISCUSSION

We have found that some of the residual neurons and most of the reactive astroglia in the hippocampus of MTS patients expressed Bcl-2 immunopositivity. Because Bcl-2 is not expressed in the normal adult hippocampus (8,31–33) induction of the antiapoptotic protein Bcl-2 may indicate an ongoing survival attempt in mildly injured neurons as also suggested by reports showing increases in growth factor and heat shock protein-70 expression in hippocampi of patients with MTS (34,35). Increased Bcl-2 immunostaining in reactive astroglia may be related to a nonspecific glial proliferative response to brain injury, as it also was reported for various brain pathologies associated with reactive gliosis (36,37). Conversely, Bax immunoreactivity was increased in the majority of the affected hippocampal neurons compared with the control hippocampal sections. Although immunohistochemistry has shortcomings for quantitative comparisons, these findings are in accordance with the results of immunoblotting studies performed on frozen specimens from MTS patients (9). The enhanced Bax expression in the affected hippocampal neurons may indicate an increased potential for activation of the apoptotic cascade in these cells (13,38). Presence of fractin immunopositivity in ~10% of neurons strongly suggests that downstream steps in the apoptotic cascade also were activated in some of these neurons. The fractin antibody used recognizes the 32-kDa actin fragment generated after cleavage of actin filaments by caspases. It does not crossreact with the intact actin or the actin fragments produced by calpains; therefore it is considered to be a reliable marker of caspase activation (26). Supporting the idea of fragmentation of the cytoskeletal protein actin in these cells, the fractin-positive neurons had a shrunken morphology.

Rapid developments in the field have recently disclosed the basic molecular machinery involved in apoptosis (13,38). In the well-established mitochondrial pathway, interactions between pro- and antiapoptotic members of Bcl-2 family appears to play a critical role in making the life/death decision (19). Once the death decision is reached, apoptotic dismantling of the cell is triggered by setting free the apoptotic protease-activating factor (Apaf-1), which activates the executioner caspases that will cleave several cytoplasmic and nuclear proteins to cause cell death (13,38,39). Unlike random DNA strands breaks seen in necrosis, DNA strands are precisely cleaved at internucleosomal linker points in apoptosis, exposing 3′-OH ends that can be detected by TUNEL staining (15,16). However, we could not disclose DNA fragmentation in hippocampus specimens of patients with MTS despite evidence indicating increased Bax expression and subsequent caspase activation. A methodologic flaw is unlikely because we routinely used a control section from human medulloblastoma specimens, which stained positively in every trial. Moreover, in three other studies conducted in our laboratory, human cortical specimens obtained from subacute sclerosing panencephalitis (SSPE) patients (40) and mice brains subjected to ischemia (27,28) all were stained positively with the same batches of the ApopTag kit. TUNEL staining was negative, and Hoechst-33258 staining did not disclose characteristic nuclear features of apoptosis in degenerating cells in the hippocampus. Henshall et al. (9) also did not detect TUNEL positivity in most of the patients they examined except the two, of whom only one had MTS. These two patients had only a small number of cells positively stained with TUNEL. The small discrepancy between the two studies may have originated from the differences in tissue processing (18) or from differences in patient characteristics (e.g., some patients may have an accelerated rate of cell death because of recent frequent seizures) (41).

Hippocampal sclerosis is characterized by extensive loss of dentate hilar neurons and hippocampal pyramidal cells (1,2,42,43). The severity of cell loss relates to age at onset of seizures, suggesting a slowly developing cell-death process (44). No inflammatory reaction is found. These features are reminiscent of the apoptotic cell death shown in rodent models of epilepsy induced by kainic acid, pilocarpine, or repetitive perforant pathway stimulation (3–7). Increased expression of bcl-2 family gene products, caspase activation, and DNA fragmentation were all demonstrated after recurrent and prolonged seizure activity in these models (3,4,45–47).

The apparent discrepancy in TUNEL staining between MTS and experimental models of epilepsy may be caused by the rate of cell death. Cell death is likely to develop at a higher rate in a shorter period in kainite–pilocarpine or repetitive stimulation-induced epilepsy than in human temporal lobe epilepsy. Cells degenerating in small numbers over a time in MTS may be rapidly removed in the final stage by the unsaturated phagocytic capacity of the tissue and, hence, may be difficult to catch, whereas the optimal timing to capture the clusters of

TUNEL-positive cells before being cleared away from the tissue can be easily achieved in experimental models (16). TUNEL-positive neurons, a change in the Bax/Bcl-2 ratio, and fractin positivity also have been reported in Alzheimer's disease, in which a slow rate of neuronal death is also estimated (26,32,37,48,49). However, controversies continue with regard to the significance of TUNEL staining, and some authors suspect that premortem metabolic disturbances or delays in tissue processing might have caused the TUNEL positivity (20,50). A similar discrepancy also has been observed between the experimental models of Parkinson's disease and brain specimens from parkinsonian patients (51–54). Although the rate of cell death may be a significant factor determining the probability of detecting TUNEL-positive cells, it also is possible that neuronal apoptosis in MTS may be realized with an incomplete sequence of the classic form (e.g., without DNA fragmentation) or with caspase-3–independent (TUNEL-negative) DNA degradation (55–57). Interestingly, the phenotype of neuronal death appears to be complicated after acute insults such as global and focal ischemia. In ischemia, several markers of apoptosis including TUNEL staining, increased Bax/Bcl-2 ratio, activation of caspase-3 and -8, and fractin positivity have been convincingly demonstrated (14) but characteristic morphologic features of apoptosis could not be found with electron microscopy (58–60). Similarly, neuronal death in MTS may have a mixed phenotype encompassing only some features of apoptosis. Future developments disclosing the mechanisms of slow neuronal degeneration and cell death with mixed phenotype may help to complete the picture for the removal process of irreparably injured neurons in MTS.

In conclusion, with the TUNEL method, we could not find histochemical evidence for internucleosomal DNA fragmentation in the temporal lobe specimens resected from patients with MTS despite prominent neuronal loss and evidence for caspase activation. These data suggest that either the full cascade of classic apoptosis is not involved in cell loss in MTS or very slow rate of cell demise may have precluded finding TUNEL-positive neurons dying by apoptosis. Increased Bax staining and detection of caspase-mediated actin cleavage in hippocampal neurons support the latter possibility. More studies are warranted to understand the cell death mechanisms in MTS because inhibition of the apoptotic mechanisms in addition to the conventional anticonvulsant treatment may decrease cell loss in the hippocampus and positively affect progression of the disease.

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