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CELL DEATH AND SURVIVAL MECHANISMS ARE CONCOMITANTLY ACTIVE IN THE HIPPOCAMPUS OF PATIENTS WITH MESIAL TEMPORAL SCLEROSIS

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Abstract—Mesial temporal lobe epilepsy (MTLE) is often characterized pathologically by severe neuronal loss in the hippocampus. In this study we investigated concomitant appearance of the pro-apoptotic and anti-apoptotic mechanisms in injured neurons in epileptic human hippocampi. Postsurgical hippocampal specimens of randomly selected 25 patients with MTLE were studied with standard immunohistochemical techniques to detect the below markers of cell death pathways: truncated Bid – tBid, mitochondrial translocation of Bax (markers of pro-apoptotic Bcl-2 protein activation) and nuclear translocation of AIF (caspase-independent pro-apoptotic pathway). For cell survival pathways, we investigated the expression of c-IAP1, c-IAP2 and Hsp70 (heat shock protein). Immunopositive cells were counted in different regions of the hippocampus. We also verified IAP (inhibitor of apoptosis) expression with Western blotting. The results were statistically compared with hippocampi from non-epileptic autopsy controls. In patient hippocampi, Bax and tBid immunoreactivity were significantly increased and Bax staining was consistent with mitochondrial translocation. AIF was not translocated to the nucleus. c-IAP1 and c-IAP2 were barely detectable in control hippocampi, whereas their expression was dramatically increased in the patients in all hippocampal subfields. Interestingly, these neurons were also positively co-labeled for tBid and translocated Bax. Hsp70 immunreactivity was significantly increased in all surviving neurons in patient hippocampi whereas degenerating neurons failed to express Hsp70. Our findings are consistent with both pro-apoptotic and anti-apoptotic mechanisms being active within the same hippocampal neurons of patients with MTLE, illustrating an ongoing struggle between cell death and survival mechanisms in neurons under stress.

Key words: epilepsy, mesial temporal sclerosis, apoptosis, hippocampus, c-IAP.

INTRODUCTION

Hippocampal sclerosis (HS) is the most common lesion found in patients with refractory temporal lobe epilepsy (TLE) and is present in 60–70% of the cases referred for surgical resection. Removal of the mesial temporal structures eliminates seizures in up to 80% of the cases, which argues that HS may be necessary for the generation of seizures. The mechanisms that lead to the typical pattern of HS are still poorly understood (Cavazos et al., 1994; Represa et al., 1995). Repeated seizures have been implicated as the cause of progressive damage and neuronal loss in vulnerable areas such as the hippocampus (Kalviainen et al., 1998; O'Brien et al., 1999; Fuerst et al., 2003). Although the exact molecular events responsible for cell death following seizures remain unknown, experimental studies in animal models of epilepsy and human brain tissue have revealed a role for apoptotic cell death pathways (Henshall and Simon, 2005). Generally, initiation of the cell death pathway requires either activation of surface-expressed death receptors (extrinsic pathway) or release of apoptosis-activating factors from the mitochondria (intrinsic pathway), both of which lead to apoptosis in a caspase-dependent manner (Hengartner, 2000). There is also a caspase-independent pathway, where mitochondrial apoptosis inducing factor (AIF) translocates to the nucleus and activates specific DNAses therein. So far, few studies have investigated apoptotic cell death pathways in the brain tissue obtained from epileptic patients. Available evidence suggests that intrinsic as well as extrinsic pathways may be involved in apoptotic cell death in this group of patients (Nagy and Esiri, 1998; Henshall et al., 2000, 2004; Uysal et al., 2003; Shinoda et al., 2004; Schindler et al., 2006; Yamamoto et al., 2006b), whereas AIF does not seem to be a contributor (Schindler et al., 2006).

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Abbreviations: H&E, hematoxylin and eosin; HS, hippocampal sclerosis; Hsp, heat shock proteins; IAP, inhibitor of apoptosis proteins; KA, kainic acid; MTLE, mesial temporal lobe epilepsy; tBid, truncated Bid; TLE, temporal lobe epilepsy.

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Despite considerable cell loss, many neurons retain viability especially in the CA2 sector and the dentate gyrus. Unlike the pro-apoptotic pathways, the anti-apoptotic cell survival mechanisms in ''epileptic'' human hippocampi are not well characterized. Few studies have investigated the role of anti-apoptotic Bcl-2 family members, Bcl-XL (Henshall et al., 2000) and Bcl-2 (Henshall et al., 2000; Uysal et al., 2003), demonstrating a significant increase in their levels. However, there is lack of information about the role of inhibitor of apoptosis proteins (IAP) family members and heat shock proteins (Hsp), the other well-known apoptosis inhibitors.

In this study, we aimed to investigate cellular co-localization of the pro-apoptotic and anti-apoptotic pathways in hippocampal neurons of patients with HS. Since activation of the extrinsic and intrinsic apoptotic pathways is well documented in the hippocampus of these patients, we sufficed with immunohistochemical detection of the cellular distribution of truncated Bid (tBid) for activation of the extrinsic pathway, and mitochondrial translocation of constitutively expressed cytoplasmic Bax to monitor the intrinsic pathway. We also investigated whether the caspase-independent mechanisms (i.e. AIF) were activated in degenerating hippocampal neurons. For cell survival mechanisms, the expression of c-IAP1 and c-IAP2, two members of the IAP family, and Hsp70, a ubiquitous molecular chaperone that acts as an inhibitor at multiple levels of the apoptotic cascade, were investigated.

EXPERIMENTAL PROCEDURES

Subjects

This study was performed on hippocampal specimens obtained from 25 patients (16 F, 9 M; age: 15–49, mean 28 ± 9 years) who underwent resective surgery for drug-resistant mesial temporal lobe epilepsy (MTLE) in our Epilepsy Center. The study protocol was reviewed and approved by the Hacettepe University Ethics Committee, and an informed written consent was obtained from each subject. All the patients underwent continuous scalp video-EEG monitoring. Interictal and ictal EEG findings and seizure semiology were consistent with temporal lobe seizures. High resolution (1.5–3 T) cranial MRI disclosed unilateral hippocampal atrophy and hyperintensity, indicating HS. None of the patients had any other co-existing lesions. Neuropsychological tests were performed in all and the WADA test was performed preoperatively, when necessary. Clinical and laboratory findings were discussed at a weekly conference where a consensus was reached upon the final diagnosis and treatment strategy. All patients underwent standard anterior temporal lobectomy with amigdalohippo-campectomy.

Methods

All the resected tissue specimens were consistent with HS and only those where anatomical orientation was possible were included in the study. Control hippocampal sections, which displayed no macro- or microscopic abnormalities, were obtained from five adult autopsy cases. Additionally, surgically removed hippocampal specimen from one patient was left at room temperature for 6 h before processing, in order to ensure that any potential alterations in autopsy controls would not be due to delay in postmortem tissue fixation.

Tissue preparation

En bloc hippocampal resections were fixed in 10% formalin before routine processing in paraffin. Five-μm-thick serial coronal sections, passing through the body of the hippocampal formation were obtained and processed routinely. Sections were stained with hematoxylin and eosin (H&E) and with antisera against mitochondrially translocated Bax (Desagher et al., 1999; Hartmann et al., 2001), tBid, AIF, c-IAP1, c-IAP2 and Hsp70. All slides were examined by a neuropathologist (F.S.) and two neurologists (N.D. and T.D.) separately and together. Human glioblastoma tissue was used as a positive control for Western blot analysis of c-IAP1 (Karmakar et al., 2006). The specimen was frozen in liquid nitrogen immediately after removal and kept at −80 °C until processing.

Immunohistochemistry

We used standard immunohistochemical procedures to stain all sections. Antigen retrieval was applied to all antibodies except Hsp70. Briefly, sections were pressure cooked in 0.1 M citrate buffer for 3 min and then were immunostained with anti-Bax (rabbit polyclonal; Santa Cruz; 1/250 dilution), anti-tBid (rabbit polyclonal; Biosource; 1/100 dilution), anti-AIF (goat polyclonal; Santa Cruz; 1/100 dilution), anti-c-IAP1 (rabbit polyclonal; Santa Cruz; 1/50 dilution) and anti-c-IAP2 (rabbit polyclonal; Santa Cruz; 1/50 dilution) antibodies. The same procedures, except antigen retrieval, were applied for Hsp70 immunostaining (mouse monoclonal; Sigma; 1/100 dilution). All procedures were performed at room temperature. The primary antibody for Bax was incubated at room temperature for one hour, whereas the rest was incubated overnight at +4 °C. The conventional avidin–biotin–peroxidase technique was carried out and DAB was used as a chromogen. Nuclei were visualized by hematoxylin or methylene green counterstaining. Positive and negative controls were included in each experiment. Importantly, control and HS sections were processed simultaneously to ensure standard staining conditions. The sections were coverslipped with mounting solution. Anti-AIF-immunostained sections were also coverslipped with mounting solution containing Hoechst-33258 (H-3569; Molecular Probes). For colocalization of the pro- and anti-apoptotic markers within the same cell we stained two successive 5-μm-thick hippocampal slices from the same patient with anti-Bax or anti-cIAP1 antibodies each ($n = 3$ patients). Double immunostaining of the same section could not be preformed since both antibodies were rabbit polyclonal.

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-0.6×) and a digital camera (Nikon DXM 1200) at a resolution of 1280 × 1024 pixels. The UV-2A (EX: 330–280, DM: 400; BA: 4209) filter was used for viewing Hoechst-33258 fluorescence. Digital images were fed to a computer and processed with ACT-1 (version 2) image-processing software provided by the manufacturer (Nikon). For sections immunostained with anti-AIF antibody, light microscopic and fluorescence images were obtained from the same sections, which were captured and digitized as indicated earlier and then were superimposed to study nuclear staining in neurons.

The percentage of neurons positively stained with the antibodies (stained neurons/counted neurons × 100) was assessed under high-power (400×) magnification in six different hippocampal sub-fields (1-CA1; 2-CA2; 3-CA3; 4-hilus; 5-dentate gyrus and 6-subiculum) in both the study group and autopsy controls. The findings in Bax, tBid, c-IAP1 and