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The Role of *bcl-2* Family of Genes During Kindling

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Summary: *Purpose:* Several experimental models of human temporal lobe epilepsy have shown that apoptotic death of neurons is an important part of this degenerative disease. However, the role of apoptotic regulators is not clear during the epileptogenesis. Therefore we investigated the expression pattern of *bcl-2* family of genes during the formation of kindling model of epilepsy in rats.

Methods: We examined the expression pattern of *bax, bcl-2, bcl-x_L, mtd,* and *bcl-w* both at messenger RNA (mRNA) and protein level in the brain tissues during the formation of epilepsy with kindling model in adult rats, which has been the most acceptable form of experimental model of human epilepsy. We also assessed the onset of DNA fragmentation by using the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay.

Results: Animals have started to have epileptic discharges after day 10 of kindling model. Recurrent subthreshold electrical stimuli induced not only epileptic foci but also the expression of *bax*, an inducer of apoptosis, in this time period. Conversely, *bcl-x_L*, which is an inhibitor of apoptosis, had an opposite pattern of expression both at mRNA and protein level during the formation of epilepsy. We did not observe DNA fragmentation by TUNEL staining.

Conclusions: Our study shows differential expression of Bax and Bcl-x_L at the CA1 region during the formation of hippocampal kindling model. The absence of DNA fragmentation during this period suggests that epileptic changes in neurons have the potential to induce DNA fragmentation by altering the expression levels of Bax and Bcl-x_L. **Key Words:** Apoptosis—Kindling—Bcl-2 family of genes—Epilepsy—Rat.

Selective neuronal loss in hippocampus is frequently associated with human temporal lobe epilepsy (1–3). Exitotoxicity-induced necrosis has long been thought to be the mechanism of neuronal damage during the induction of epilepsy (4–7). However, several recent studies also implied that neurons die via apoptosis after such an injury (8–12). Apoptotic death of neurons also has been shown in several experimental models of human temporal lobe epilepsy including kindling (10,12).

In kindling model of epilepsy, repeated subconvulsive electrical stimulation eventually results in generalized seizures (13,14). Postmortem analysis of humans with chronic idiopathic epilepsy suggests that neuronal damage is cumulative and related to the frequency of seizures (15). Therefore the kindling model of epilepsy seems to possess several features that may be related to clinical problems of epilepsy. By using this experimental model, it has been shown that administration of protein synthesis inhibitor, cyclohexamide, prevented the onset of apoptosis in brain, suggesting that protein synthesis is required in this process (10).

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Apoptosis has been crucial for normal development of several systems including nervous system. Extensive studies performed over the last 10-year period revealed a large part of the molecular basis of apoptosis made it clear that the disruption in apoptotic pathways results in many pathologic conditions. Therefore the molecular regulators of the apoptosis have a vital importance in living organisms. Among these, Bcl-2 family of proteins determines the life or death of a cell by controlling the release of mitochondrial apoptogenic factors, cytochrome *c*, and apoptosis-inducing factors (AIFs), which activate the downstream executional phases, including the activation of the caspases (16,17). The *bcl-2* family of genes consists of both pro- and antiapoptotic genes, and by forming dimers, they exert their function. The ratio of death antagonists (Bcl-2, Bcl-x_L) to agonists (Bax) has been shown to have a critical role in determining the fate of the cells (18,19). The Bcl-2 family proteins also are key candidates in contributing to seizure-induced neuronal death outcome. Alterations within the Bcl-2 family of proteins, including Bax, Bcl-2, Bcl-X_S, Bcl-X_L and Bcl-w, have been shown both in human temporal lobe epilepsy (20–22) and in different experimental murine models of epilepsy (12,23–32). Among these, by using the amygdala-kindled model, Zhang et al. (12) found that the mRNA

expression of *bax* but not *bcl-2* was increased in rat brains after kindled seizures (12).

However, to our knowledge, the participation of the *bcl-2* family of genes has not been investigated during the formation period of the kindled form of epilepsy. In addition, it is not clear whether this neuronal death is directly involved in epileptogenesis or occurs secondary to the effects of the severe and prolonged seizures. To elucidate the role of *bcl-2* family of genes and apoptosis during epileptogenesis, we investigated the expression pattern of the different *bcl-2* genes both at mRNA and protein level, by monitoring at different stages of the kindling model of epilepsy in rats. Our observations provide insight into the involvement of the Bcl-2 family of proteins during epileptogenesis.

METHODS

Animals

Male, 9-week-old, 280- to 300-g Sprague–Dawley rats were used. They were housed under controlled environmental conditions (22°C) with a 12-h light and 12-h dark cycle in the animal holding facility of the Department of Molecular Biology and Genetics at the Bilkent University, Turkey. All the animals received care according to the criteria outlined in the "Guide for Care and Use of Laboratory Animals" prepared by the National Academy of Science, and this study protocol complied with Bilkent University's guidelines on humane care and use of laboratory animals. The animals were permitted unlimited access to food and water at all times. Before used in the experiments, the rats were allowed to adapt the new conditions for $\geq$1 week. Animals were randomly divided into three groups: one group was implanted with electrodes and received electrical impulses, the other group of animals was implanted with electrodes but did not receive electrical impulses and served as a sham group, and the last group of animals was neither implanted with electrodes nor stimulated with electrical impulses, and thus served as a control group. Animals were killed 1, 3, 7, 10, and 14 days after the electrical impulse (n = 3 at each time point) with corresponding sham and control groups of animals.

Kindling procedure

Rats were anesthetized by using phenobarbital (PB; 50 ml/kg, i.p.) and placed in a stereotaxic frame. A bipolar, plastic-coated stainless-steel electrode was implanted into the right ventral hippocampal CA1 region at the following coordinates: tooth-bar at 0: 4.8 mm caudal to bregma, 5.2 mm lateral to midline, and 6.5 mm ventral to dura (33). A reference electrode was placed between the skull and left temporal muscle. Ten days after surgery, we started to give one kindling stimulation (1-s, 100-Hz biphasic pulses of 1-ms duration, 400 μA peak-to-peak amplitude delivered by a stimulator) every day for 14 days. Animals were killed 1, 3, 7, 10, and 14 days after the initial stimulus. Behavioral seizures were scored as follows (34): grade 0, normal behavior, wet-dog shakes, arrest; 1, facial twitches; 2, head nodding, chewing; 3, forelimb clonus; 4, rearing, falling on forelimbs; 5, falling on the side or back, hindlimb clonus. Electroencephalographic activity was monitored by a Nihon-Kohden 14-channel EEG recorder.

Tissue fixation, synthesis of sense and antisense mRNAs, and in situ hybridization protocols

Rats were killed and their brains were quickly removed and cryopreserved by putting them into 4% cold paraformaldeyde for 18 h and then into 30% sucrose for 24 h. The full-length rat *bax, bcl-2, bcl-w, mtd,* and *bcl-x_L* were cloned into pGEM4Z (Promega, Madison, WI, U.S.A.) and pBluescript II SK$^+$ (Stragene, La Jolla, CA, U.S.A.) expression vectors. After linearization with the appropriate restriction enzymes, digoksin-labeled antisense or sense RNAs were synthesized by using the Dig RNA labeling kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. After ethanol precipitation, the probes were stored at –70°C until used. The 15-μm cryosections were placed on the Silane-treated slides and were hybridized with 10 ng digoksin-labeled *bax*, *bcl-2, bcl-w, mtd,* and *bcl-x_L* probe at 30 μl hybridization buffer consisting 40% deionized formamide, 10% dextran sulfate, $\times$1 Denhardt's solution (0.002% Ficoll, 0.02% polyvinylpyrrolidone, 10 mg/ml Rnase-free bovine serum albumin), $\times$4 SSC, 10 m*M* DDT, 1 mg/ml yeast t-RNA, and 1 mg/ml denatured and sheared salmon sperm DNA. Sense and antisense probes were placed on the upper and lower half of every slide, respectively, and separate coverslips were used for these sections. Slides were placed horizontally in a sealed, humidified container and incubated at 42°C for 16 h; then the coverslips were removed, and slides were washed with $\times$2 SSC and $\times$1 SSC for 15 min each and subjected to 30-min incubation at 37°C with NTE buffer (500 m*M* NaCl, 10 m*M* Tris, and 1 m*M* EDTA, pH 8.0) containing 20 μg/ml Rnase A to digest any single-stranded RNA probe. To visualize the bound probes, slides were then incubated with AP-labeled anti-digoksin antibody at 1:500 dilution at 4°C for 16 h. Slides were then incubated with NBT/BCIP as substrate with 10 m*M* levamisole to block endogenous AP activity. Then the slides were examined by using a Zeiss-Axioskop microscope at $\times$4, $\times$10, and $\times$40 magnification. To confirm tissue integrity, some slides were subjected to hematoxylin/eosin staining and examined under bright-field illumination.

Immunocytochemistry

Cryosections (5 μm) were incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous

peroxidase activity. After washing 3 times with phosphate-buffered saline (PBS) for 10 min each, slides were incubated with preblocking serum (normal goat serum, 1.5%; bovine serum albumin, 2%; Triton-X, 0.1%) for 1 h at room temperature. The primary antibody of Bax, Bcl-x_L, Bcl-2, Bcl-w, and Mtd (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) was applied at a concentration of 1 μg/ml in preblocking solution and kept at 4°C overnight. After washing 3 times with PBS, tissue sections were incubated for 1 h with 3 μg/ml of biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, U.S.A.) in the preblocking solution containing 1% normal rat serum (Sigma), washed in PBS, and incubated for 1 h with an avidin-biotin complex reagent containing horseradish peroxidase (HRP) (Vector Laboratories) in PBS. After washing 10 min with PBS, slides were rinsed in 0.5% Triton-X 100/PBS for 30 s. Color development was achieved by incubation with diaminobenzidine (DAB) solution (Vector Laboratories) for 7 min. The tissues were examined by using Zeiss-Axioskop microscope at ×4, ×10, and ×40 magnification.

To analyze immunoreactivity, the number of immunopositive cells in sections was semiquantitatively scored (35). The scoring was as follows: 0, not present (Fig. 4D); 1, light (Fig. 4C, F, G, H, J); 2, moderate (Fig. 4A, E, I); 3, high (Fig. 4B).

In situ analysis of DNA fragmentation (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling; TUNEL)

DNA fragmentation was detected in situ with a TdT- (terminal deoxynucleotidyl transferase)–mediated fluorescein-dUTP labeling kit (Roche Diagnostics, Mannheim, Germany). Brain cryosections were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then washed in PBS for 30 min. After incubating with a permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice, 50 μl of TUNEL reaction mixture was put into each sample and incubated for 1 h at 37°C in the dark in a humidified chamber. Slides were then directly analyzed with fluorescence microscopy. For evaluation by fluorescence microscopy, we used an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm. As negative control, we incubated the slides in the absence of TdT. For positive controls, the samples were first treated with DNase I (1,000 U/ml in 50 m*M* Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 min at 20°C to induce DNA strand breaks before labeling procedures and then incubated with 50 μl of TUNEL reaction mixture.

RESULTS

Induction of epilepsy in animals (kindling procedure)

To investigate the participation of the *bcl-2* family of genes during the formation period of kindled epilepsy, first we performed the kindling procedure to induce epilepsy. After day 10, we observed stage 4 kindling seizures (rearing and falling on forelimbs) as defined by Racine (34). As seen from EEG traces, afterdischarges were evident after applying electrical stimulus on day 1 (Fig. 1A). We observed epileptic EEG activity on day 10 (Fig. 1B) and day 14 (Fig. 1C). We did not detect any epileptic activity in the sham and control group of animals in their EEG traces (data not shown).

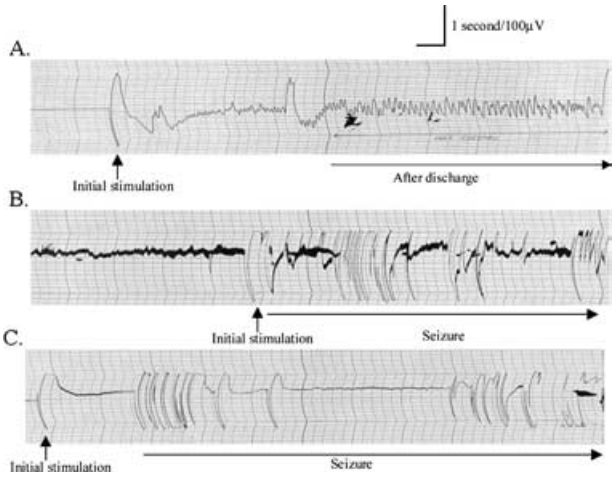

FIG. 1. Patterns of electrographic activity during kindling. EEG traces were recorded from the electrically stimulated animals on day 1 **(A)**; day 10 **(B)**; and day 14 **(C)**. After day 10, we observed stage 4 kindling seizures (rearing and falling on forelimbs).

To elucidate the role of the *bcl-2* family of genes and apoptosis during epileptogenesis, we investigated the expression pattern of different *bcl-2* family of genes (*bax, bcl-x_L bcl-2, mtd, bcl-w*) at both the mRNA and protein levels, by monitoring at different stages of the kindling model of epilepsy in rats by using in situ hybridization, immunohistochemistry, and in situ analysis of DNA fragmentation (TUNEL).

bax mRNA expression

We observed *bax* mRNA expression in the hippocampal CA1 area as early as 1 day after the electrical stimulus (Fig. 2A). In the corresponding sham group of animals on day 1, *bax* mRNA expression also was present but to a lesser extent (Fig. 2B). Decreased expression in the sham group suggests that the increase in the expression of *bax* is due to electrical stimulus. Increased mRNA expression of *bax* was observed on day 9 (data not shown), but it was more evident at day 10 (Fig. 2C). Similar to day 10, *bax* expression persisted on day 14 (data not shown). Conversely, *bax* expression was not detectable in the sham group of animals on day 10 (Fig. 2D). By using sense *bax* mRNA as a control, we did not detect any specific binding (Fig. 2E). We also observed morphologic changes in the limbic lobe tissues starting on day 10 (Fig. 2C). These changes were not present in sham group animals on day 10

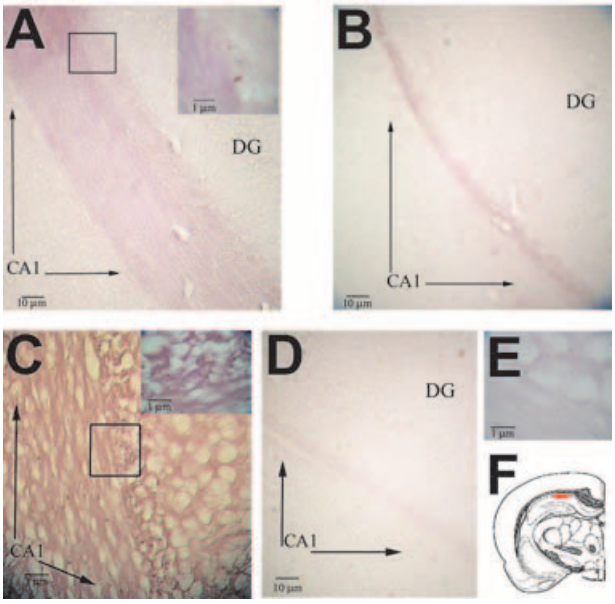

FIG. 2. The expression of *bax* mRNA in CA1 region **(F)** during the formation of kindling. The expression of *bax* mRNA was examined by using antisense **(A–D)** and sense **(E)** *bax* riboprobes on day 1 **(A)** and day 10 **(C)** of electrically stimulated animals and compared to that of sham group on day 1 **(B)** and day 10 **(D)**. **A:** *bax* mRNA expression was present in CA1 region of electrically stimulated rat brains on day 1. *Inset, bax* mRNA-positive and -negative staining area shown at high power. **B:** Decreased expression of *bax* mRNA in CA1 region of sham groups on day 1. **C:** *bax* mRNA expression increased on day 10 of electrically stimulated rat brains. *Inset, bax* mRNA-positive and -negative staining area shown at high power. **D:** No detectable *bax* mRNA expression in CA1 region of sham groups on day 10. **E**: Section probed with sense *bax* riboprobes revealed no specific binding. **F:** Cross section of brain at 35th plate, red rectangle denotes CA 1 region. *Arrows*, indicate the CA1 region in the brain tissues. DG, dentate gyrus.

(Fig. 2D), which rules out the local irritation of implanted electrodes.

bcl-x$_L$ mRNA expression

We found similar expression levels of *bcl-x$_L$* mRNA at the CA1 region both in the electrically stimulated (Fig. 3A) and the sham group of animals (Fig. 3B) on day 1. This suggested that a basal level of expression existed, and this expression was not affected by electrical stimulus. Interestingly, when we examined the later stages, *bcl-x$_L$* expression almost diminished on day 8 (data not shown) and day 10 of electrically stimulated animals (Fig. 3C). In contrast, on day 10, a similar level of *bcl-x$_L$* expression was present in both sham (Fig. 3D) and control groups (data not shown) compared with day 1. By using sense *bcl-x$_L$* mRNA, we did not detect any specific binding (Fig. 3E). The expression of *bcl-x$_L$* was absent on day 14 in the electrically stimulated animals (data not shown).

bcl-2, mtd, and *bcl-w* gene expression

We did not detect any modulation in the expression of these genes in our in situ hybridization experiments (data not shown).

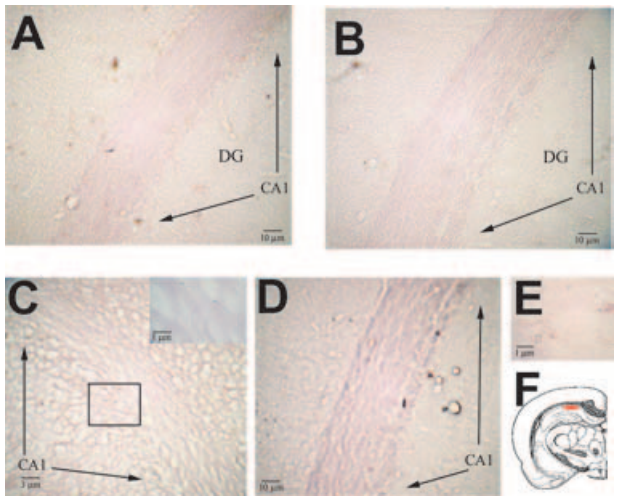

FIG. 3. The expression of *bcl-x$_L$* mRNA in CA1 region **(F)** during the formation of kindling. The expression of *bcl-x$_L$* mRNA was examined by using antisense **(A–D)** and sense **(E)** *bcl-x$_L$* riboprobes on day 1 **(A)** and day 10 **(C)** of electrically stimulated animals and compared to that of sham group on day 1 **(B)** and day 10 **(D)**. **A:** *bcl-x$_L$* mRNA expression was detected in CA1 region of electrically stimulated rat brains on day 1. **B:** Similar levels of the expression of *bcl-x$_L$* mRNA in CA1 region of sham groups on day 1. **C:** *bcl-x$_L$* mRNA expression was not detectable on day 10 of electrically stimulated rat brains. *Inset, bcl-x$_L$* mRNA-negative staining area shown at high power. **D:** The expression of *bcl-x$_L$* mRNA was present in CA1 region of sham groups on day 10. **E:** Section probed with sense *bcl-x$_L$* riboprobes revealed no specific binding. **F:** Cross section of brain at 35th plate, red rectangle denotes CA 1 region. *Arrows* indicate the CA1 region in the brain tissues. DG, dentate gyrus.

Bax protein expression

We observed increased Bax protein expression on day 10 (Fig. 4B) compared with day 1 (Fig. 4A) with respect to electrically stimulated animals. Bax expression persisted on day 14 (data not shown). However, in the sham animals, a very light expression was noted on day 1 (Fig. 4C), and no expression was seen on day 10 (Fig. 4D). In electrically stimulated animals, the expression level of Bax on day 7 (Fig. 4I) was less than to that of day 10 but more than that of day 1. No differences were seen in the expression of control animals between days 1 and 10 (data not shown). No staining was seen in negative controls (data not shown).

Bcl-x$_L$ protein expression

Similar to its mRNA expression, we observed decreased Bcl-x$_L$ protein expression in the stimulated groups of animals on day 10 (Fig. 4F) compared with day 1 (Fig. 4E). Conversely, in the sham groups of animals, the expression levels were similar on days 1 (Fig. 4G) and 10 (Fig. 4H). Compared with Bax expression, an opposite pattern of expression in Bcl-x$_L$ was observed on day 7 of group of electrically stimulated animals. The level of Bcl-x$_L$ expression on day 7 (Fig. 4J) was less than that of day 1 but more than that of day 10. No expression of Bcl-x$_L$ was observed on day 14 in the electrically stimulated animals

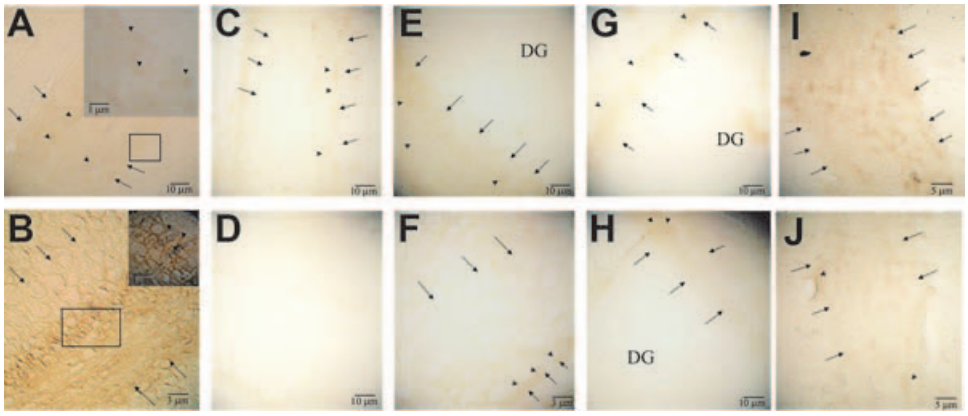

FIG. 4. The expression of Bax **(A–D, I)** and Bcl-x_L **(E–H, J)** protein in CA1 region during the formation of kindling. The expression of Bax on day 1 **(A)** and day 10 **(B)** of electrically stimulated animals, compared to that of sham group on day 1 **(C)** and day 10 **(D)**. Bax protein expression increased on day 10 in electrically stimulated animals. The expression of Bcl-x_L protein on day 1 **(E)** and day 10 **(F)** of electrically stimulated animals compared to that of sham group on day 1 **(G)** and day 10 **(H)**. The levels of Bcl-x_L protein was decreased in the stimulated group on day 10, whereas in the sham groups, there was no difference between the expression on day 1 and day 10. To illustrate the effect of electrical stimulation on the expression of these proteins during kindling, the level of Bax and Bcl-x_L proteins was also assessed on day 7 (**I** and **J**, respectively). *Arrows* indicate the CA1 region in the brain tissues. *Arrowheads* indicate immune reactive cells. DG, dentate gyrus.

(data not shown). No differences were seen in the expression of control animals between days 1 and 10 (data not shown). To assess the specific binding, no expression was observed when the primer antibody was omitted (data not shown).

Bcl-2, Mtd, and Bcl-w protein expressions

We did not find any changes in the expression levels of Bcl-2, Mtd, and Bcl-w protein in our groups (data not shown).

In situ analysis of DNA fragmentation (TUNEL)

Increased Bax and decreased Bcl-x_L expression both in mRNA and protein levels during kindling suggested the presence of apoptosis, because the ratio between these genes has been altered to favor apoptotic death. Therefore we investigated the onset of apoptosis by examining the DNA fragmentation. However, we did not detect any TUNEL-positive cell in any of the day 1 and day 14 groups (Fig. 5A and B). Conversely, when we treated the tissue with DNase I to induce DNA strand breaks, as a positive control, we found many TUNEL-positive cells (Fig. 5C).

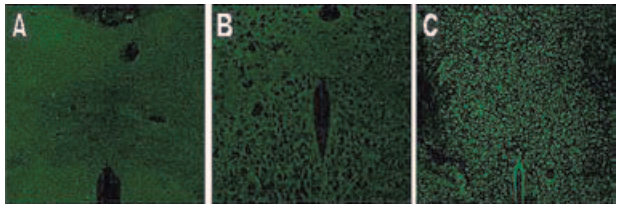

FIG. 5. Assessment of the presence of DNA fragmentation by TUNEL during the formation of kindling. No positive cells was seen on day 1 **(A)** and day 10 **(B)**. On the other hand, there were many TUNEL-positive cells when the sections were treated with DNase I to induce DNA breaks **(C)**.

DISCUSSION

This study characterized the expression pattern of the *bcl-2* family of genes at both mRNA and protein levels during epileptogenesis in a highly successful experimental murine model of kindling. By using this animal model, previously Zhang et al. (12) observed an increased ratio of *bax*/*bcl-2* mRNA expression and apoptosis in the hippocampus of adult rats after the formation of seizures. In this study, we went one step beyond and investigated the involvement of the *bcl-2* family of genes at both the mRNA and protein levels during the formation process of epileptic seizures by using in situ hybridization, immunohistochemistry, and in situ analysis of DNA fragmentation (TUNEL) experiments.

Abnormal excitability has been identified in neurons or synapses in multiple sites in the kindled brain by electrophysiologic analyses of in vitro brain slices; these sites include the dentate granule cells, CA3 and CA1 pyramidal cells of the hippocampus, pyramidal neurons of the pyriform cortex, and neurons in the basolateral nucleus of the amygdala (36–39). The amygdala, which requires relatively little stimulation to induce kindling, is a particularly convenient structure because of its large size, simplifying the stereotaxic placement of a stimulating/recording electrode (39). In our study, we preferred to stimulate particularly the CA1 region to have a prolonged period of kindling to observe early apoptotic events more easily during epileptogenesis.

Extensive studies performed over the last 10 years have revealed considerable information on the molecular basis of apoptosis. The mitochondria play an essential role in the apoptotic death of mammalian cells by releasing various apoptogenic proteins, including cytochrome *c*, into the cytoplasm (16,17). The Bcl-2 family of proteins

regulates these mitochondrial changes during apoptosis. One of the major function of the Bcl-2 protein family is to control membrane permeability directly, although the precise mechanisms by which Bcl-2 family members do so are still to be determined. It has been proposed that the ratio of the expression within the members of this family is critical in the fate of a cell; whether it should live or undergo programmed cell death (18,19). Therefore we chose to detect the expression levels of *bax, bcl-2, bcl-x_L, bcl-w*, and *mtd* among the *bcl-2* family of genes.

Alterations within some of the Bcl-2 family of proteins have been shown both in human temporal lobe epilepsy (20,21) and in different experimental murine models of epilepsy (12,23–32). Our results provide evidence that although subthreshold electrical stimuli generate an epileptic focus during kindling, they also cause differential *bax* and *bcl-x_L* gene expression in CA1 region, resulting in disruption of the delicate balance within the expression of the *bcl-2* family of genes. During the formation of the kindling procedure, the expression of *bax* increases, whereas *bcl-x_L* expression decreases at the hippocampal CA1 region, at both mRNA and protein levels. Particularly interesting is that the balance between the expression of Bax and Bcl-x_L is shifted in favor of Bax on day 10 of the kindling procedure. Detection of Bcl-x_L but not Bax expression in the corresponding sham and control group of animals further supports this contention. This finding implies that neuronal morphologic or functional changes or both due to epileptogenesis could be responsible for triggering apoptotic events. However, new studies are warranted to show the physical interaction of Bax-Bcl-x_L, such as co-immunoprecipitation.

We also observed that electrically stimulated animals developed a distinct histologic profile 10 days after the first electrical stimulus. The intensity of the current that we used during kindling formation was very low and, as defined by Engel (40), this low intensity of electricity while inducing kindling does not result in histologic damage. In addition, electrical impulses in the earlier days (before day 10) did not cause this histologic appearance. Not observing this appearance in the sham group of animals rules out local irritation due to implants. The epileptic discharges that caused stage 4 kindling seizures in these animals may be responsible for this appearance by changing the metabolic activity of the epileptic tissue. It is known that when epileptic discharges continue for a long time, morphologic changes in the tissue can be induced. Moreover, functional loss due to the epileptic discharge (Todd paresis) after a focal epileptic seizure is an indicator of the presence of histopathologic changes in the tissue (40). Tissue edema, which is considered one such change, may be associated with the appearance that we observed after epileptic seizure. Despite the lack of studies regarding to histopathologic changes after kindling seizures, this view also could be related to increased hippocampal volume (41,42).

Internucleosomal DNA fragmentation is considered to be a biochemical hallmark of apoptosis. Several studies have used this simple method in a wide variety of CNS disorders to identify apoptotic cells (43). However, we did not detect any DNA fragmentation with our TUNEL assay within our groups. One explanation for the absence of DNA fragmentation in our experimental system might be that we observed an early period of apoptosis. Because DNA fragmentation is a late event in the apoptotic processes, TUNEL negativity does not necessarily exclude a stage of the initial apoptotic process. Observing the TUNEL positivity in our positive control group also rules out the possibility of a methodologic flaw. Alternatively, other members of the Bcl-2 family of proteins may interact with Bax or Bcl-x_L or both to prevent the release of cytochrome *c* from mitochondria, which ultimately blocks the activity caspases, and thus the onset of apoptosis. Nevertheless, the functional importance of cytochrome *c* in kindled rats is not known.

In addition, TUNEL negativity may be related to the intracellular localization of Bax protein. It is known that Bax is localized in the cytoplasm, and on receiving a death signal, it is translocated to the mitochondria and starts a cascade of events resulting in the release of cytochrome *c* (44), leading to apoptosis. The translocation of Bax protein also has been shown to participate in neuronal cell death (45). It may be possible that Bax is not translocated into the mitochondria because of its interaction with a molecular chaperone protein 14-3-3, which negatively regulates the activity of Bax (46). Furthermore, another member of the Bcl-2 family of protein, Bad, has been shown to displace Bax from Bcl-x_L, resulting in the translocation of Bax to the mitochondria (25). Therefore in our experimental system, Bad may be one of the factors responsible for the lack of DNA fragmentation. Studies to assess protein–protein interaction dynamics and dimerization responses of Bcl-2 family proteins are required to assess the role of these proteins properly during the kindling process.

In summary, we investigated the expression levels of the Bcl-2 family of proteins during the formation of kindling epileptogenesis and found that differential expression of Bax and Bcl-x_L at CA1 region accompanied the formation of the epileptic focus. Because these modulations occurred before the onset of apoptosis, it can be concluded that epileptic changes in neurons have the potential to induce apoptosis through regulation of Bax and Bcl-x_L. Better understanding of this regulation may result in new genetic treatments to prevent the formation of epilepsy.

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