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Differential Expression of Rat Brain Bcl-2 Family Proteins in Development and Aging

Shun Shimohama,* Sadaki Fujimoto,† Yasuo Sumida,† and Hiroko Tanino†
*Department of Neurology, Faculty of Medicine, Kyoto University, Sakyoku, Kyoto 606, Japan; and
†Department of Environmental Biochemistry, Kyoto Pharmaceutical University, Kyoto 607, Japan

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We have previously examined the involvement of the B cell leukemia-2 gene product (Bcl-2) family proteins (Bcl-2, Bcl-x, Bax, Bak, and Bad) in Alzheimer's disease (AD) and found that Bcl-2, Bcl-x, Bak, and Bad were upregulated. As AD is an aging-associated disease, in the present study we examined the developmental and aging-related changes in Bcl-2 family proteins in the rat brain. Immunoblot analyses of brain extracts from embryonic day 19 (E19) to postnatal 96-week-old rats indicated that the Bcl-2 protein level was highest at E19 and decreased after birth. Bcl-x levels remained high from E19 to 96 weeks. Bax levels were high from E19 to 2 weeks and decreased from 4 weeks onward. Bak levels were highest at E19 and decreased abruptly after birth. Bad levels were high from E19 to 2 weeks and decreased abruptly at 4 weeks. The present results suggest that the expression of each Bcl-2 family protein is differentially regulated during development and aging and that the changes in the senescent brains are different from those observed in AD.

Recent studies on apoptosis suggest that neuronal selection during both embryonic development and some forms in adults is regulated by members of the B cell leukemia-2 gene product (Bcl-2) family such as Bcl-2, Bcl-x, Bax, Bak and Bad (1–3). Bcl-2 prevents apoptotic death in neurons (4) and several neuron-like cell lines (5). Moreover, overexpression of Bcl-2 in transgenic mice protects neuronal cells from naturally occurring cell death and experimental ischemia (6). Bcl-x also prevents excessive neuronal cell death in development (7, 8); Bcl-x-deficient mice die around embryonic day 13, with extensive apoptotic cell death evident in postmitotic immature neurons (9). In contrast, Bax, Bak and Bad promote apoptosis (1–3), probably by forming heterodimers with Bcl-2 or Bcl-x, and thereby abolishing their protective function (2, 3, 10, 11).

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the progressive deterioration of cognitive function and memory in association with widespread neuronal cell death (12–14). In a previous study we found that the levels of Bcl-2, Bcl-x, Bak and Bad were increased in the particulate fraction of the temporal cortex of autopsy brains from patients with AD (15).

As AD is an aging-associated disease, it is important to consider such alterations in the context of normal developmental and aging-associated changes in apoptosis-regulating proteins, but these are little understood. In the present study we examined the developmental and aging-related changes in Bcl-2 family protein levels in the cerebral cortex from embryonic (E19) to postnatal 96-week-old Wistar rats, and found a unique and differential expression of each Bcl-2 family protein during development and aging.

Abbreviations used: AD, Alzheimer's disease; Bcl-2, B cell leukemia-2 gene product, BSA, bovine serum albumin; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

MATERIALS AND METHODS

Materials. Primary antibodies included: mouse monoclonal antibodies to Bcl-2 (B46620), Bcl-x (B22620), and Bad (B31420) from Transduction laboratories (Lexington, KY); rabbit polyclonal antibody to Bax (P-19) from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal antibody to Bak (clone TC100, AM03) from Oncogene Research Products (Cambridge, MA). An enhanced chemiluminescent detection system (ECL kit) from Amersham (Buckinghamshire, England) and Vectastain ABC Elite kit from Vector Laboratories (Burlingame, CA), were used for immunodetection. Other chemicals were of reagent grade and were obtained commercially.

Brain samples. Wistar rats pregnant for 19 days (E19) were anesthetized under ether inhalation, and rat fetuses were removed from the uteri by Caesarian section. Brains were taken from E19 fetuses as well as from 1-, 2-, 4-, and 8-week-old male Wistar rats purchased from Japan SLC (Kyoto). Twenty-four-, 36-, 48-, 72-, and 96-week-old male Wistar rats were bred in our laboratory. The animals were treated in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Preparation of brain extracts. Brain tissue samples from the cerebral cortex (1 g wet wt) were homogenized with a Teflon–glass homogenizer in 4 vol of 10 mM Hepes buffer (pH 7.0) containing 0.32 M sucrose, 0.05% NaN_3, 100 μM orthovanadate, 0.1 mM phenyl-

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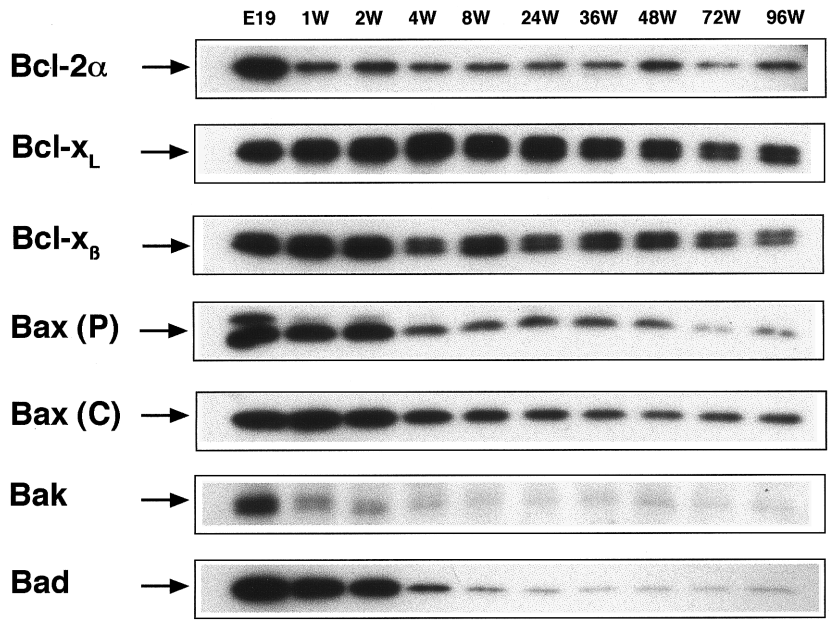

FIG. 1. A typical demonstration of the immunodetection of Bcl-2, Bcl-x, Bax, Bak, and Bad in development and aging. Particulate (Bcl-2α, Bcl-x_L, Bax, Bak, and Bad) and cytosolic fractions (Bcl-x_β) from the cerebral cortex of Wistar rat brains were loaded (10 μg protein/lane), and immunoblot assays were performed using antibodies against these proteins. E19, embryonic day 19; W, postnatal week.

methylsulfonylfluoride, 0.5 mM diisopropylfluorophosphate, 1 mM dithiothreitol, 10 μg/mL aprotinin, 5 μg/mL pepstatin A, 5 μg/mL leupeptin, 5 mM benzamidine, and 4 mM ethylene glycol tetraacetic acid. The homogenate was centrifuged at 105,000*g* for 60 min and the supernatant thus obtained was used as the cytosolic fraction. The pellet was washed twice, suspended in homogenization buffer, and used as the particulate fraction.

Immunochemical detection. Protein concentration was measured by the method of Bradford (16). Proteins in the particulate and cytosolic fractions from developing and aging rat brains, suspended in Laemmli sample buffer, were subjected to 4–20% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE), and blotted onto Immobilon (Millipore, Bedford, MA). The Immobilon membrane was incubated with Tris-buffered saline (pH 7.5) containing 0.3% Triton X-100 (TBS-T) and 5% dehydrated skim milk (Difco Laboratories, Detroit, MI) to block nonspecific protein binding. The membrane was then incubated with primary antibodies including mouse monoclonal antibodies to Bcl-2 (diluted 1:500), Bcl-x (1:500), Bad (1:500), or Bak (1:400), or rabbit polyclonal antibody to Bax (diluted 1:300) followed by secondary antibody, horseradish peroxidase (HRP)-linked antibodies against either mouse or rabbit immunoglobulin (each diluted 1:1000). Subsequently, bound HRP-labeled antibodies were detected by chemiluminescence (ECL kit, Amersham). Protein bands reacting with antibodies were detected on radiographic film (X-Omat JB-1, Kodak) 5 to 60 s after exposure. The integrated optical density for the protein band recognized by each antibody was measured by scanning densitometry (Arcus II, Agfa, Germany) in order to assess the relative quantity of each Bcl-2 family protein. To test if Bcl-2, Bcl-x, Bax, Bak, and Bad immunostaining on the blots was linear within the protein range examined, their immunoreactivity in samples containing 5–50 μg of protein was measured and plotted versus protein. All of these graphs were linear for protein values between 5 and 50 μg ($r = 0.90$ to 0.99, respectively) (data not shown). Prestained SDS–PAGE standards (Bio-Rad, Richmond, CA) were used as molecular weight markers. The apparent molecular weight of phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme was 112, 84, 53.2, 34.9, 28.7, and 20.5 kDa, respectively, according to the manufacturer's instructions. Results are given as mean ± standard error of the mean (SEM).

RESULTS

Immunoblots of Bcl-2 Family Proteins in the Particulate and Cytosolic Fractions of Rat Brain

In the cerebral cortex of rat brains, 27-kDa Bcl-2α was detected abundantly in the particulate fraction, but not in the cytosolic fraction. The antibody to Bcl-x identified a doublet of proteins with an apparent molecular weight of 29/30 kDa (Bcl-x_L) mainly in the particulate fraction. On the other hand, a 27-kDa Bcl-x_β-like protein was detected chiefly in the cytosolic fraction. Twenty-one-kiloDalton Bax was identified in both the particulate and cytosolic fractions, whereas Bak and Bad proteins, with apparent molecular weights of 30 and 23 kDa, respectively, were identified mostly in the particulate fraction (data not shown).

Developmental and Aging-Related Changes in Bcl-2 Family Protein Levels in the Rat Cerebral Cortex

Bcl-2. The protein level of Bcl-2α was highest at E19, decreased remarkably after birth, and remained low from 4 to 96 weeks of age (Fig. 1 and Fig. 2A).

Bcl-x. The protein level of Bcl-x_L in the particulate fraction increased from E19, being highest at 2 weeks, and remained high during development and aging (Fig. 1 and Fig. 2B). Bcl-x_β in the cytosolic fraction was at high levels from E19 to 4 weeks of age, peaking at 2 weeks, and remained moderately high from 8 to 96 weeks (Fig. 1 and Fig. 2C).

Bax. The protein level of Bax in the particulate fraction was highest at E19, remained high until 2

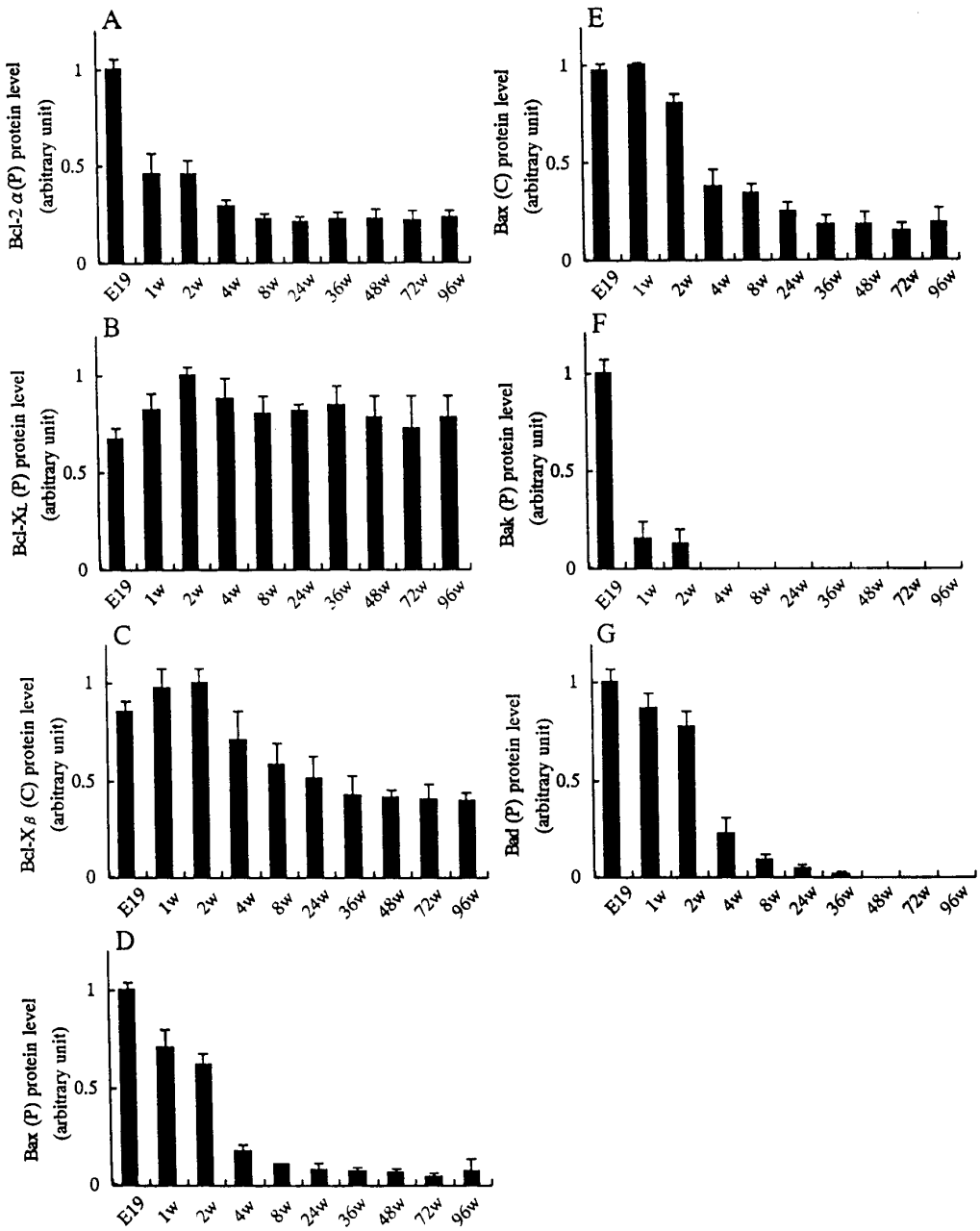

FIG. 2. Developmental and aging-related changes in the levels of Bcl-2, Bcl-x, Bax, Bak, and Bad in the rat cerebral cortex. (A) Bcl-2α protein level in the particulate fraction. (B) Bcl-x_L protein level in the particulate fraction. (C) Bcl-x_β protein level in the cytosolic fraction. (D) Bax protein level in the particulate fraction. (E) Bax protein level in the cytosolic fraction. (F) Bak protein level in the particulate fraction. (G) Bad protein level in the particulate fraction. The method of immunochemical detection is described under Materials and Methods. Bars indicate the standard error of mean (SEM) (n = 6).

weeks, and then abruptly decreased from 4 weeks onwards (Fig. 1 and Fig. 2D). Bax in the cytosolic fraction was at high levels from E19 to 2 weeks, then decreased, and remained low from 4 to 96 weeks of age (Fig. 1 and Fig. 2D).

Bak. Bak was highly expressed at E19, minimally detected at 1 and 2 weeks, and barely detected after 4 weeks (Fig. 1 and Fig. 2F).

Bad. The protein level of Bad was highest at E19, decreasing gradually after birth until 2 weeks, and abruptly decreasing at 4 weeks (Fig. 1 and Fig. 2G).

DISCUSSION

The present study demonstrated a differential and unique expression of apoptosis- regulating Bcl-2 family

proteins in the rat brain during the course of development and aging.

Bcl-2 was expressed highly during embryonic development but was downregulated after birth. Naturally occurring cell death in the developing rat cerebral cortex, which increases during the first postnatal week and decrease thereafter, disappearing by the end of the first month, is found to have the characteristics of apoptosis and is associated with endonuclease activation (17). During this process, neurons expressing Bcl-2 may determine whether a neuron dies or survives, as over-expression of Bcl-2 in transgenic mice protects neurons from naturally occurring cell death (6). However, it is also true that low levels of Bcl-2 protein are present in the adult and aged brain. The sustained expression of this anti-apoptosis protein may protect neurons from various injuries or neurodegeneration. In addition, recent studies suggest that Bcl-2 can serve as a marker of both proliferating and differentiating neurons and that such immature neurons may be much more widespread in postnatal primate brain than previously thought (18). In contrast, Bcl-x_L expression in the particulate fraction and Bcl-x_β expression in the cytosolic fraction were maintained at a high level postnatally in the brain, suggesting that it may play an important role in the regulation of neuronal survival in the adult and aged brain (7). Continued neuronal survival after Bcl-2 is downregulated may require persistent Bcl-x expression in addition to target-derived neurotrophic factors produced in the formation of appropriate synapses (19).

Bax, Bak and Bad promote apoptosis, probably by forming heterodimers with Bcl-2 or Bcl-x, and abolishing their protective function (1–3, 10, 11). The present study indicated that Bax protein is present in both the particulate and cytosolic fractions but that the expression profile in each fraction during development and aging is slightly different. Bax in the particulate fraction was expressed highly in the embryo, moderately in the early postnatal period, and little after 4 weeks of age, an expression pattern similar to that of Bcl-2α. Bax in the cytosolic fraction, however, was expressed highly in the embryonic and early postnatal periods, and moderately high during adulthood and aging, an expression profile similar to that of Bcl-x_β, suggesting that Bax may form heterodimers with Bcl-2α in the particulate fraction and with Bcl-x_β in the cytosolic fraction.

Little is known about the expression of Bak and Bad during the development and aging of the brain. Recently, Obonai *et al.* (20), in an immunochemical study, showed that Bak expression in the cerebrum and cerebellum is high in the brains of human fetuses and elderly subjects, but low in those of young adults, and suggested that Bak regulates neuronal death associated with the development and aging of the brain. Their results are, in part, consistent with ours in that Bak expression in the brain is high in the fetus and absent from infancy to adolescence, but inconsistent in that Bak was not expressed even at 96 weeks of age in the rat brain. This discrepancy might be due to species difference, although more control human samples might be necessary to clarify the expression of Bak in the aged brain. Until now no data have been available regarding the expression profile of Bad during brain development and aging. The present study has clarified that the expression of Bad continued later than that of Bak in the rat brain, which suggests a different role for Bad and Bax in naturally occurring cell death during development.

The expression profiles of Bcl-2 family proteins strongly suggest that Bcl-2α, Bcl-x, Bax, Bak, and Bad are involved in naturally occurring cell death in the developing rat brain. In contrast, the cell death process associated with neuronal senescence remains to be elucidated. Senescence is associated with an increase in the production of DNA fragments during brain oxidative stress, which probably leads to more necrosis and apoptosis than in the younger brain (21). In consequence of this hypothesis, for decades it has been widely accepted that neuron death in the neocortex and hippocampus is an inevitable result of brain aging, but recent quantitative studies have suggested that neuron death is restricted in normal aging (22). The results from the present study showed that Bcl-2 and Bcl-x which prevent apoptotic neuronal death, and Bax, Bak, and Bad, which promote apoptosis, were neither up- nor downregulated during aging, which would occur around 96 weeks of age, suggesting that aging-related apoptosis dose not occur in senescence. In AD, Bcl-2α, Bcl-x, Bak and Bad were remarkably upregulated (15), suggesting that neurodegenerative events underlying AD may be distinct from the events that mediate age-related impairment. This view also suggests that age-related impairment and AD are not a continuum, and that the former does not necessarily reflect a predisposition for the latter.

Though the role of apoptosis in the maintenance of physiological tissue homeostasis during embryonic and fetal development is clearly defined, several pathological conditions and external factors also cause apoptosis. Reexpression of apoptosis-promoting Bak and Bad in AD may characterize the neuronal or nonneuronal cells of the brain that precede cell death in AD. In addition, upregulation of Bcl-2 and Bcl-x may imply that those neurons involved in AD can synthesize a range of molecules enabling them to adapt rapidly and successfully to a cell death-threatening environment (23). Therefore, it might be important to explore the extrinsic and intrinsic regulators that modulate the expression of Bcl-2, Bcl-x, Bak, and Bad in a positive or negative direction in order to improve the efficacy of anti-AD treatment.

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