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Differential Expression of Neuronal Death-related Factors in Aged Rat Cerebellum

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In the present study, we investigated the expression of apoptosis-associated proteins in the cerebellum of aged rats: IGF-I receptor (IGF-IR), nitrotyrosine (NT), p53, key pro-apoptotic gene ICH-1 (caspase-2), c-Fos and Bcl-2 family members (Bcl-2 and Bax).

Twelve adult (4～6 month old) and 15 aged (24～29 month old) Sprague-Dawley rats were examined in this study. We performed immunohistochemical staining, *in situ* hybridization and densitometric measurement using a NIH image program (Scion Image) to determine the staining density.

In adult rats, there were no immunoreactivities for insulin-like growth factor-I receptor (IGF-IR), nitrotyrosine (NT) or p53 in any region of cerebellum. However, IGF-IR immunoreactivity was found in some Purkinje cells in aged rat cerebellum. The prominent staining of NT or p53 was also localized in the Purkinje cell layer in aged rats. A high density of ICH-1 (caspase-2) immunoreactivity was observed in the molecular and Purkinje cell layers in aged rats. Immunoreactivity for c-Fos was significantly decreased in the granule cells in aged rats. Positive signal for bcl-2 was significantly decreased in the Purkinje cells and granule cells of aged rats. The most intense staining for Bax was observed in the soma of Purkinje cells of adult rats. However, Bax immunoreactivity was not changed in any layers in the cerebellar cortex of aged rats.

In conclusion, this study provides the first morphological data concerning the differential regulation of apoptosis-related genes in rat cerebellum during aging.

Key words : Apoptosis-associated factors, Aging, Cerebellum, Immunohistochemistry, *In situ* hybridization

Introduction

Apoptosis (programmed cell death) plays an indispensable role in the development and maintenance of homeostasis (Vaux and Korsmeyer 1999). The key pro-apoptotic gene is the interleukin-1β converting enzyme (ICE) (Yuan et al. 1993), a cysteine protease with specificity for cleavage after aspartate residues (Thornberry et al. 1992). Subsequent studies revealed the presence of the ICE/Ced-3 related proteases that are now referred to as caspases, including Nedd2/Ich-1 (caspase-2) (Wang et al. 1994) and CPP32 (caspase-3) (Fernandes-Alnemri et al. 1994). Constitutive expression of high levels of Bcl-2 protein enhances survival of many cell types including neurons on exposure to various adverse stimuli. Bcl-2 forms heterodimer with a related protein, Bax, which has been suggested to oppose the anti-apoptotic function of Bcl-2

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(Oltvai et al. 1993). Hydroxy radicals-induced apoptosis was associated with the decrease in bcl-2 mRNA level and the increase in the protein levels of c-Fos (Xin et al. 2000). The bcl-2 and Bax gene has been shown to exhibit activation in response to increased p53 levels, consistent with the identification of p53-responsive elements in the Bax promoter (Miyashita and Reed 1995).

The insulin-like growth factor (IGF-I) has been shown to have anti-apoptotic and neuroprotective activity, promoting survival of cerebellar neurons (Baker et al. 1999). Specific regions of the IGF-I receptor (IGF-IR) are responsible for the anti-apoptotic activity of IGF-I and a number of studies have demonstrated a link between these anti-apoptotic effects and the pro-survival Bcl-2 proteins. In a neuronal hyperosmotic stress model, apoptosis was blocked by activation of the IGF-IR with a 4-fold decrease in Bcl-2 and correlated with processing of caspases (Singleton et al. 1996). Chrysis et al. (2001) have demonstrated that IGF-I decreases caspase-3 availability and activity, increases the expression of anti-apoptotic Bcl-x_L and Bcl-2 during early postnatal development, and decreases proapoptotic Bax and Bad expression at later developmental stages.

The cerebellum is known to be critically involved in age-related loss of balance and fine control, and cerebellar damage is known to yield motor impairment. Apoptosis is a common pathway for Purkinje cell death in pathological conditions such as some human cerebellar ataxias (Ikeda et al. 1996). In ataxia (*ax*) mice (Ohgoh et al. 2000) and leaner mice (Fletcher et al. 1996), TUNEL-positive cells were detected in the granular layer and occasionally in Purkinje cells. In Down syndrome, enhanced apoptosis may be involved in disease-related neuronal loss (De la Monte et al. 1998). Recently, Engidawork et al. (2001) have demonstrated that in Alzheimer's disease (AD), the frontal cortex and cerebellum are equally affected in apoptosis. Although there is evidence that these apoptosis-related genes participate in the regulation of differentiation, physiological or pathological cell death, the involvement of these genes in the aging process has not yet been established in cerebellum. Therefore, the present study used *in situ* hybridization and immunohistochemical staining to investigate the involvement of apoptosis-associated proteins in the cerebellum of aged rats: Bcl-2 family members Bcl-2 and Bax; key pro-apoptotic gene ICH-1 (caspase-2); IGF-IR that are responsible for the anti-apoptotic activity of IGF-I; oxidative damage marker nitrotyrosine (NT); regulator protein p53, which is essential for cell-cycle arrest and induction of apoptosis as a response to chromosomal damage; and c-Fos that may implicate a kind of cellular stress response in the aging process. Our results show differential alterations of these cell death-regulatory proteins, and suggest their potential roles in neuronal degeneration and death in aged cerebellum.

Materials and Methods

1. Subjects and tissue preparation

Twelve adult (4～6 month old) and 15 aged (24～29 month old) Sprague-Dawley rats were examined in this study. The animals used in this experiment were treated according to the 'Principles of Laboratory Animal Care' (NIH publication No. 86-23). The animals were anesthetized with sodium pentobarbital (60 mg/kg). They were perfused transcardially with cold phosphate buffered saline (PBS, 0.02M, pH 7.4), and then with ice-cold 4% paraformaldehyde for 10 min at a flow rate of 50～60 mL/min. Brains were removed immediately and sliced into blocks 4～6 mm thick. These were immersed in a cold fixative for 6～12 hrs and then washed in a series of cold sucrose solutions of increasing concentrations. Frozen sections were cut at 40 μm in the coronal plane. For in situ hybridization, the animals were anesthetized with sodium pentobarbital (60 mg/kg). They were sacrificed by decapi-

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tation and the brains were quickly removed and frozen rapidly with 2-methyl butane. Sections (12 μm) on gelatin-coated slides were made on a cryostat.

2. Immunohistochemistry

The sections were incubated using the free-floating method for 48～72 hrs at 4°C in primary antiserum containing Triton X-100 (0.3%), bovine serum albumin (0.5 mg/mL) and normal goat serum (3 drops/10 mL), and the appropriate dilution of the primary antibody: monoclonal IGF-IR antibody (1 : 4,000; Calbiochem, Cambridge, MA), ICH-1 (1 : 100; Transduction Laboratory, Lexington, KY), c-Fos polyclonal antibody (1 : 500; Calbiochem), rabbit anti-rat Bax polyclonal antibody (1 : 2,000; Pharmingen, San Diego, CA), mouse monoclonal anti-NT antibody (1 : 2,000; Upstate biotechnology, Lake Placid, NY) and mouse anti-rat p53 monoclonal antibody (Dako, Glostrup, Denmark). Sections were visualized according to the avidin-biotin complex (ABC) method, using an ABC kit (Vectastain™, Vector Laboratories, Berlingame, CA), and then developed for peroxidase reactivity with 3, 3′-diaminobenzidine (DAB) (Sigma, St. Louis, MO). A sample of sections was reacted without primary antiserum, and a different sample was exposed to primary antiserum that had been preincubated for 24 h with each corresponding protein. No sections from both groups exhibited any immunoreactivity described in this report. Sections from each adult and aged group were stained together eliminating conflicts between different experimental conditions.

3. *In situ* hybridization

The sections were fixed, acetylated, dehydrated and treated with chloroform for protein removal. PTRI-Bcl-2-Mouse (Ambion, Austin, TX) was used as the templates for preparation of bcl-2 cRNA probes. A digoxigenin (DIG)-11-UTP (Boeringer Mannheim, Mannheim, Germany) labeled antisense cRNA probe was generated by T7 RNA polymerase (Promega, Madison, WI). Hybridization was performed with 350 ng/mL antisense or sense probes at 42°C overnight. After incubation with anti-DIG-alkaline phosphatase (BM), NBT (160 μg/mL, Promega) and BCIP (330 μg/mL, Promega) were dropped on the slides, and the reaction was stopped with distilled water when color development was thought to be adequate.

4. Evaluation of immunostaining

We selected five slides in each area of the adult (n=12) and aged rats (n=15), and counted all of the immunoreactive neurons in each corresponding area of the brain. Visual assessment and densitometric measurement using a NIH image program (Scion Image) determined the staining density. Student t-test was performed to investigate whether age-related changes were statistically significant (*$p < 0.01$).

Results

In adult rats, no IGF-IR-immunoreactive cells were found in any region of cerebellum (Fig. 1A). In contrast, IGF-IR immunoreactivity was found in some Purkinje cells in aged rat cerebellum. The most prominent immunolabeling in cerebellar cortex was localized within the cell bodies and dendrites of Purkinje cells (Fig. 1B). Immunoreactivity for NT, a neurochemical marker for oxidative reactions, was also found in Purkinje cells only in aged rats, which is similar with the localization of IGF-IR (Fig. 1C). The immunoreactive labeling of p53 in aged cerebellar cortex was localized in the cell bodies of Purkinje cells and dendrites in molecular layers (Fig. 1D), whereas there was no immunoreactivity above background level in adult rats.

In adult rat cerebellum, there was a much lower density of ICH-1 in the molecular and Purkinje cell layers compared to aged rats (Fig. 1E). At a higher

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magnification, ICH-1 immunoreactivity was detected in the cell bodies and dendrites of Purkinje cells in the cerebellar cortex (Fig. 1F). The cerebellar output neurons showed moderate immunoreactivity for ICH-1 in the nucleus lateralis, interpositus and medialis of aged rats, with relatively weaker staining in the surrounding neuropil (Fig. 1G). In adult rats, there was a high density of c-Fos in the granular layer with a much lower density of c-Fos in the molecular and Purkinje cell layers (Fig. 1H). At a higher magnification, c-Fos immunoreactivity was detected in the Purkinje cell bodies, not in the dendrites. In aged rats, c-Fos immunoreactivity was significantly decreased in the granule cells (Fig. 1I).

In the cerebellum of adult rats, the most intense signal for bcl-2 was observed in the soma of Purkinje cells and granular layer, with a relatively weaker signal in the molecular layer (Fig. 2A, B). Granule cells were positively stained with an intensity similar to that noted in the Purkinje cell layer. Basket and stellate cells in the molecular layer exhibited moderate signals. Signals were not observed in the white matter. In aged rats, bcl-2 positive signals were significantly decreased in the Purkinje cells and granule cells (Fig. 2C, D). There were no signals in the molecular layer of aged rats. At a higher magnification, a decrement in bcl-2 positive signals was prominent in the cerebellar cortex with aging.

The most intense staining for Bax was observed in the soma of Purkinje cells, but did not extend into the dendrites in adult rats (Fig. 2E). Moderate staining was observed throughout the molecular layer, which included the round cell bodies of basket and stellate cells. Granule cells were uniformly stained with an intensity similar to that noted in the molecular layer. In the cerebellar cortex of aged rats, Bax immunoreactivity was not changed in any layers (Fig. 2F). In adult deep cerebellar nuclei, Bax immunoreactivity was observed in the large cell bodies of cerebellar output neurons (Fig. 2G). In addition to the cell bodies, Bax immunoreactivity was found in the surrounding neuropil, the region where the Purkinje cell axons terminate. In aged rats, the cell bodies of cerebellar output neurons showed moderate immunoreactivity for Bax, with relatively weak staining in the processes (Fig. 2H). In image analysis, mean density of Bax immunoreactivity was not significantly changed in the cerebellar nuclei of the aged rat.

Discussion

The present study demonstrated spatial and temporal expression patterns of neuronal death-related genes in rat cerebellum during normal aging (Table 1). Several intracellular proteins have been identified in the IGF anti-apoptotic cascade including members of the caspases and the Bcl-2 proto-oncogene family (Toms et al. 1998). Jung et al. (1996) have shown that caspase mediated cell death in COS cells is suppressed by IGF-I without affecting the expression levels of Bcl-2, Bcl-xL or Bax. However, Singleton et al. (1996) have shown that IGF-I prevents apoptotic death of neuronal cells by a mechanism involving up-regulation of the pro-survival Bcl-2 proteins. In addition, Park et al. (1996) showed that death of PC12 cells, induced by serum deprivation, is blocked by Bcl-2 overexpression or by inhibitors of caspases. The present results showed for the first time that immunoreactivities for IGF-IR and ICH-1 were increased in Purkinje cells of aged rats whereas bcl-2 mRNA level was decreased in the Purkinje cell and granular layers of aged rats. The unexpected increase in IGF-IR in the cerebellum of aged rats may be an attempt by these regions to protect vulnerable neurons that have reduced trophic support.

The cerebellum might be particularly dependent on Bcl-2 since Bcl-2 knockout mice result in a marked decrease in the number of cerebellar cells and an increase in endpoints of oxidative stress (Hochman et al.

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Table 1. Changes in mean densities of cell death-regulatory proteins and bcl-2 mRNA in rat cerebellum during aging

	Layers	Adult rats	Aged rats
IGF-IR	Molecular layer	36.9±8.6	78.4±17.2*
	Purkinje cell layer	49.3±6.5	144.8±27.9*
	Granular layer	54.8±3.6	55.3±16.6
3-NT	Molecular layer	39.1±8.8	165.0±28.0*
	Purkinje cell layer	47.0±10.3	186.4±19.4*
	Granular layer	52.0±6.4	56.7±4.8
p53	Molecular layer	42.2±9.2	147.8±15.5*
	Purkinje cell layer	50.1±9.6	141.3±13.8*
	Granular layer	51.5±5.6	60.3±2.0*
ICH-1	Molecular layer	93.8±18.2	145.3±9.1*
	Purkinje cell layer	76.5±15.9	154.8±26.8*
	Granular layer	62.6±15.2	29.1±10.6*
c-Fos	Molecular layer	91.6±3.6	93.9±11.8
	Purkinje cell layer	122.8±9.3	128.3±11.0
	Granular layer	95.4±9.6	73.8±6.7*
bcl-2	Molecular layer	10.4±5.7	6.5±3.8
	Purkinje cell layer	224.0±12.3	107.8±23.4*
	Granular layer	27.1±7.7	18.0±5.8*
Bax	Molecular layer	63.0±18.6	44.9±5.6
	Purkinje cell layer	183.7±15.9	173.6±12.5
	Granular layer	94.0±17.4	71.3±4.6

The sum of the gray values of all the pixels in the selection was divided by the number of pixels within the selection. Student t-test was performed (*$p < 0.01$).

1998). In this study, Bcl-2 mRNA signals profoundly decreased in aged rats, but Bax immunoreactivity was not changed. Given the high levels of oxidative stress and the age-associated pathology in the cerebellum, apoptosis in aged cerebellum may be mediated by a Bax related antisurvival Bcl-2 protein rather than Bax itself. The decrease in Bcl-2 probably indicates the more vulnerability of cerebellar cells to apoptotic stimuli. These results may indicate that age-specific mechanisms are involved in the regulation of bcl-2 and bax expression during aging. In the cerebellum of fetal Down syndrome, the impressive finding was an at least fivefold elevation of Bax protein (Seidl et al. 2001). Together with decreased Bcl-2 values, this resulted in elevated Bax/Bcl-2 ratios in all Down syndrome cerebellar regions. In AD, however, Bcl-2, Bcl-x, Bak and Bad were remarkably upregulated (Kitamura et al. 1998), suggesting that neurodegenerative events underlying AD or Down syndrome may be distinct from the events that mediate age-related impairment.

Reactive oxygen species (ROS) exerts a regulatory role through interactions with transcription pathways and signaling pathways (Le Bourg 2001). This regulation may be altered under aging, leading to age-related degenerative diseases in CNS (Butterfield et al. 2001). Oxidative damages appear to contribute to the aging process itself (Le Bourg 2001). Bcl-2, located in mitochondrial outer membrane, is able to block apoptosis in neurons with the reduced generation of ROS (Green and Reed 1998). In the mouse brain, the amount of neuron-specific bcl-2 expression has substantially decreased by 5 months of age (Merry et al. 1994). In contrast to our major results, several studies have failed to observe decreases in Bcl-2 expression during aging. Immunoblot analyses of Wistar rats indicated that Bcl-2 expression was increased in aged F344 rats, possibly as a consequence of oxidative stress challenges (Kaufmann et al. 2001). Dorszewska et al. (2004) also demonstrated that the ratio of Bax protein to Bcl-2 protein was decreased in the cerebellum of 2-year-old rats. It is unclear whether this inconsistency reflects technical differences associated with tissue preparation or strain differences.

Oxidative damage was considered as a likely cause of age-associated brain dysfunction because the brain is believed to be particularly vulnerable to oxidative stress due to a relatively high rate of oxygen free radical generation without commensurate levels of antioxidative defense (Agarwal and Sohal 1996). An age-associated decline in motor coordination has been correlated with oxidative damage to the cerebellum (Forster et al. 1996). It has been shown that age-related deficits in motor behavior and motor learning can be prevented or reversed by high antioxidant diets in aged rat cerebellum (Bickford et al. 2000). Some of the consequences of oxidative stress are DNA modifi-

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cations, lipid peroxidation and protein modifications such as formation of NT. An increased production of superoxide and a reduced rate of turnover of the relevant tissue proteins with age might contribute to the higher NT levels detected in this study. Our first demonstrations of increased NT in Purkinje cells during aging implicate Purkinje cells as sites for functionally significant ROS-associated molecular damage.

This study demonstrated that p53 immunoreactivity was upregulated with aging. Immunocytochemistry showed intensely stained p53-immunoreactive neurons in the cerebellum of aged rat, but no p53-immunoreactive cells were observed in the control group. Several reports indicate that p53 regulates sensitivity to oxidative damage in CNS neurons (Xiang et al. 1996) and cerebellar Purkinje cells are highly sensitive to oxidative stress (Nakaso et al. 2000). Therefore, upregulation of p53 in the Purkinje cells observed in this study suggests that significant loss of Purkinje cells with aging may be regulated with several apoptosis-controlling factors including p53 and oxidative stress mechanism. Recently, Inamura et al. (2000) reported increased number of p53-positive neurons in the Purkinje cell layer using organotypic slice culture exposed to bleomycin and indicated that p53 is involved in DNA strand break-induced apoptosis of fully postmitotic central nervous system neurons.

In this study, c-Fos immunoreactivity was significantly decreased in the granular layers with aging. What are the implications of the changes of c-Fos expression in cerebellum? C-Fos expression may implicate a kind of cellular stress response, which occurs during aging. Changing c-Fos immunoreactivity in aged cerebellum may indicate its roles in cellular stress response or functional differentiation. The decrement of c-Fos expression in aged rat may correlate with functional impairment in aging, such as synaptic loss and neurochemical changes in information processing. This notion is supported by the fact that the basal expression of inducible transcription factors is indicative of neuronal plasticity in terms of reactive changes in gene expression (Herdegen et al. 1995).

In conclusion, our first demonstration showing the differential regulation of neuronal death-associated genes in aged rat cerebellum supports the contention that apoptosis may be regulated by these genes specifically during aging and neurodegeneration of cerebellum. Therefore, these morphological data may provide insights into age-related changes in substrates of differentiation or cell-death machinery as well as basic knowledge required for the study of neurodegenerative diseases such as AD.

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Legends for Figures

Fig. 1. Localizations of IGF-IR, NT, p53, ICH-1 and c-Fos in adult and aged rats. In adult rats, no immunoreactivities for IGF-IR was found in any region of cerebellum (A). In contrast, IGF-IR immunoreactivity was found in some Purkinje cells in aged rat cerebellum (B). NT immunoreactivity was also found in Purkinje cells in aged rat, which is similar with the localization of IGF-IR (C). The most prominent population of immunoreactive labeling of p53 in aged cerebellar cortex was localized within the cell bodies of Purkinje cells and dendrites in molecular layers (D). In adult rat cerebellum, there was a much lower density of ICH-1 in the molecular and Purkinje cell layers (E). At a higher magnification, ICH-1 immunoreactivity was detected in the cell bodies and dendrites of Purkinje cells in the cerebellar cortex of aged rats (F). In the interposed nuclei of aged rats, the cerebellar output neurons showed moderate immunoreactivity for ICH-1 (G). In adult rats, there was a high density of c-Fos in the granular layer (H). In aged rats, c-Fos immunoreactivity was significantly decreased in the granule cells (I). G, granular layer; M, molecular layer; P, Purkinje cell layer. Scale bar=100 μm (A, E); 50 μm (B-D, F-I).

Fig. 2. Localization of bcl-2 mRNAs and Bax protein in adult and aged rats. It was noted that bcl-2 mRNA in situ hybridization showed intensely positive signals in the Purkinje cell layer and granular layer in adult rats (A, B). In aged rats, bcl-2 positive signals were significantly decreased in the Purkinje cells and granule cells (C, D). At a higher magnification, a decrement in bcl-2 positive signals was prominent in the cerebellar cortex with aging (B, D). In the cerebellar cortex of adult rats, the most intense staining for Bax was observed in the soma of Purkinje cells (E). In aged rats, Bax immunoreactivity was not changed in any layers, compared to adult rats (F). In the cerebellar nuclei of adult rats, Bax immunoreactivity was observed in the large cell bodies of cerebellar output neurons with relatively weak staining in the processes (G). Note that mean density of Bax immunoreactivity was not changed in the cerebellar nuclei of the aged rat (H). G, granular layer; M, molecular layer; P, Purkinje cell layer. Scale bar= 250 μm (A, C); 50 μm (B, D, E-H).

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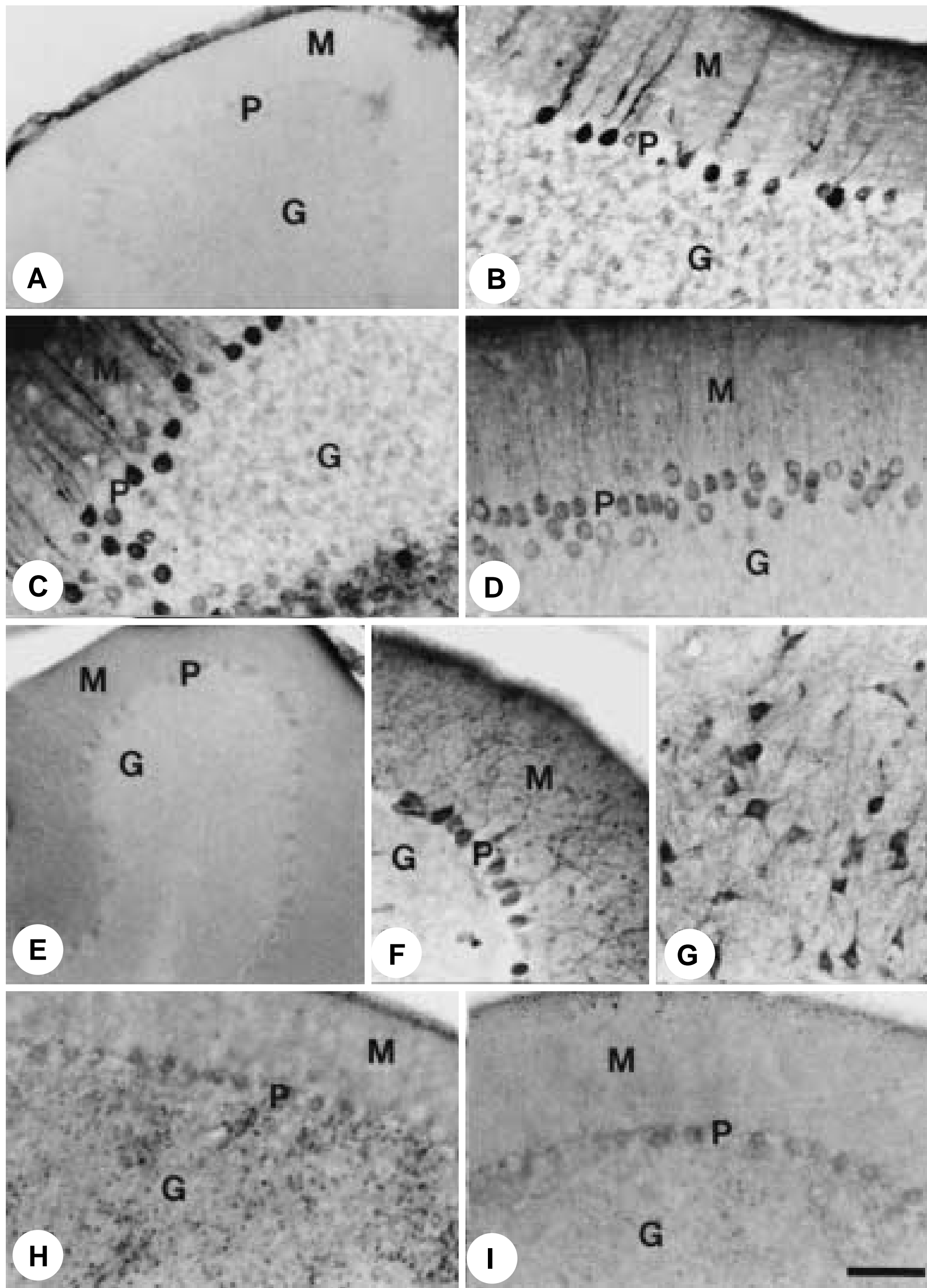

Fig. 1

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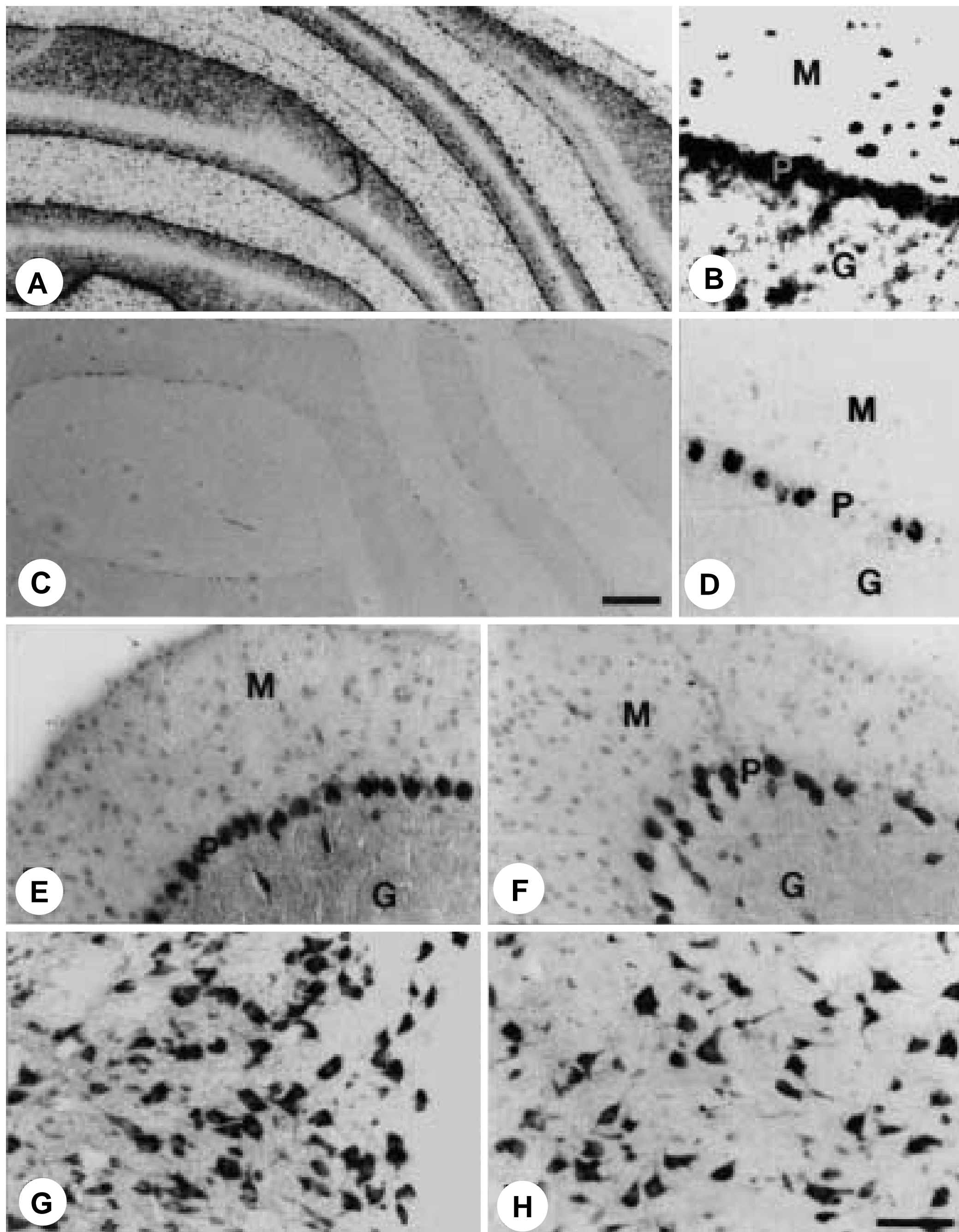

Fig. 2

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노화 흰쥐 소뇌에서 세포사 조절인자들의 변화에 대한 연구

박범준, 정윤희, 김성수, 김경용, 이원복
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간추림 : 세포자멸사는 발생과정과 중추신경계의 항상성 유지에서 중요한 역할을 하는 것으로 알려져 있다.

본 연구에서는 흰쥐 소뇌에서 노화에 따른 세포사 조절인자들의 변화를 *in situ* hybridization법과 면역조직화학법을 사용하여 관찰하였다.

TUNEL 염색 결과에서는 염색된 세포핵이 노화 흰쥐 소뇌에서만 발견되었다. IGF-IR, NT 및 p53에 대한 면역염색성은 성숙군의 소뇌에서는 거의 관찰되지 않은 반면, 노화군에서는 소뇌 겉질의 조롱박 세포에서 현저한 면역염색성을 보였다. ICH-1 (caspase-2)의 면역염색성은 노화 쥐의 분자층과 조롱박세포층에서 관찰되었다. c-Fos는 성숙군의 과립층에서 진하게 염색되었고, 면역염색성은 노화군의 과립층에서 유의성 있게 감소하였다. Bcl-2는 조롱박 세포 (Purkinje cell)층과 과립층에서 가장 강하게 발현되었고 노화에 따라 유의성 있게 감소하는 경향을 나타냈다. Bax는 성숙군의 조롱박 세포에서 가장 진하게 염색되었고, Bax 면역염색성은 소뇌 겉질과 소뇌핵에서 노화에 따른 유의성 있는 차이를 나타내지 않았다.

결론적으로, 본 연구는 흰쥐 소뇌에서 세포사 관련인자들의 노화에 따른 변화에 대한 최초의 형태학적 자료를 제공하였다. 이러한 연구는 노화를 비롯한 여러 신경퇴행성 질환 연구에 필요한 기본적인 형태학적 자료를 제공할 수 있을 것이라 사료된다.

찾아보기 낱말 : 세포자멸사 조절유전자, 노화, 소뇌, 면역조직화학법, *in situ* hybridization

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