Caspase-3 Immunoreactivity in Different Cortical Areas of Young and Aging Macaque (*Macaca mulatta*) Monkeys

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Key Words

Apoptosis • Cerebral cortex • Cerebellum • Hippocampus • Primate

Abstract

It has been shown that cytochrome-c-dependent caspase-3 activation is significantly elevated in the aging macaque brain. To assess the underlying age-related changes in the cellular distribution of caspase-3, we have examined the motor cortex, cerebellum and hippocampus of young (4-year-old, n = 4) and old (20-year-old, n = 4) rhesus monkeys by immunohistochemistry. Western blot analyses of brain homogenate showed that the antibody reacted only with inactive 32-kDa procaspase and its active 20- and 17-kDa subunits, formed after granzyme B exposure. In the motor cortex, pyramidal cells of layers III and V were moderately labeled; the underlying white matter contained weakly stained astrocytes. In the hippocampus, hilar neurons and pyramidal cells in CA3 showed the strongest immunoreaction, pyramidal cells in CA1 and granule cells of the dentate gyrus were also strongly labeled. In contrast, CA2 pyramidal cells were only weakly stained, and neurons of the molecular layer were unlabeled. Weak caspase-3 immunoreaction of CA2 neurons parallels known decreased susceptibility to

apoptosis. In the cerebellar cortex, clusters of strongly labeled Purkinje cells were observed next to groups of weakly and unstained cells; granule cells were generally unstained. The brains of aging monkeys displayed a similar pattern of caspase-3 immunoreactivity. In neocortical layer V, however, scattered very strongly labeled pyramidal cells were regularly detected, which were not observed in younger animals. This clustering of caspase-3 indicates increased vulnerability of a subset of pyramidal cells in the aging brain.

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Introduction

Apoptosis plays an important role in normal CNS development [for reviews, see 1–4] and in the pathogenesis of neurodegenerative diseases [5–10]. Caspases (cysteine-containing aspartate-specific proteases) and their substrates are pivotal in the execution of apoptosis, caspase-3 being the major effector in mammalian neuronal apoptosis [11]. Two primary pathways have been described by which caspase-3 can be activated [4, 12]. The extrinsic pathway is triggered by external cues binding to death receptors, thereby leading to the activation of caspase-8. The intrinsic pathway involves mitochondrial

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outer membrane permeabilization (MOMP) and release of proapoptotic proteins, e.g. cytochrome c. MOMP can be caused by multiple factors, including granzymes, which are released by cytotoxic T cells and NK cells to kill virus-infested and tumor cells. As a consequence, a cytosolic complex, the apoptosome, is formed activating procaspase-9. Activation of initiator caspases, either caspase-8 or caspase-9, cleaves and activates effector caspases, mainly caspase-3. Procaspase-3, the inactive precursor with a molecular weight of about 32 kDa, is cleaved to form two mature subunits, p17 (17 kDa) and p12 (12 kDa) [13], which, in turn, cleave their target proteins, resulting in the typical features of apoptotic cell death, i.e. cell shrinkage, chromatin condensation and nuclear fragmentation [12].

Deregulation of the apoptotic process in the human CNS is associated with numerous neuropathologies, including ischemic neuronal death, neurodegenerative disorders and viral infections. The majority of these diseases are age-related, and there is indication that regulation of apoptosis is altered in the aging brain [7, 14]. In order to better understand the pathogenesis of these diseases and to develop therapeutic strategies, apoptosis in the macaque brain is increasingly used as a primate animal model system [15–21]. However, relatively little is known about basic mechanisms of apoptosis in the macaque brain and even less about changes associated with aging. Assessing caspase expression in the brains of young and aging macaque monkeys, we have recently demonstrated that procaspase-9 levels are elevated in the aging brain, whereas procaspase-3 expression remains unchanged. However, cytochrome-c-dependent caspase-3 activation significantly increases with age [22]. This increase is particularly prominent in the motor cortex, where cytochrome c only elicits little caspase-3 activation in young animals, but a sixfold higher activation in older macaques.

The present study aims at localizing the cellular distribution of caspase-3 in the brains of young and aging macaques by immunohistochemistry. We want to examine if the increase in cytochrome-c-dependent caspase-3 activation observed in the aging brain is accompanied by changes in caspase-3 distribution. To characterize the antibody applied, we have also incubated monkey brain homogenates with granzyme B and determined caspase-3 expression by Western blot. We intend to assess if granzyme B is able to cleave procaspase-3 in the monkey brain and if the antibody employed reacts with inactive procaspase-3, with its cleaved subunits or with both.

Materials and Methods

Animals

All animals were treated according to *NIH Guidelines for the Care and Use of Laboratory Animals*. Experimental procedures had been approved by the ethical committees of the hospital authority in Beijing and of the Chinese University of Hong Kong. Healthy young (4-year-old, $n = 4$) and old (20-year-old, $n = 4$) female rhesus monkeys (*Macaca mulatta*), obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China, were anesthetized (ketamine hydrochloride, 10 mg/kg, i.m.) and sacrificed by exsanguination. The skull was carefully opened and brain specimens were immediately collected from the motor cortex (Brodmann's area 4), lateral hemispheres of the cerebellum and hippocampus. Tissue blocks from one half of the brain were immediately frozen in liquid nitrogen and transferred to a -80°C freezer, those from the other half were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, 4°C).

Western Blot Analysis

For preparation of brain extracts, tissue specimens (approx. 200 mg wet weight) from the three brain regions were homogenized in 4 vol of cold lysis buffer (20 mM Hepes buffer, pH 7.4, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 $\mu\text{g/ml}$ pepstatin A, 10 $\mu\text{g/ml}$ leupeptin and 10 $\mu\text{g/ml}$ aprotinin) using a Dounce homogenizer. The brain homogenate was then centrifuged at 20,000 g (30 min, 4°C). The supernatant was collected and the total protein concentration was measured with Pierce's bicinchoninic acid assay kit using bovine serum albumin as a standard (Pierce Biotechnology, Inc., Ill., USA). To identify caspase-3 in the brain, 100 μg protein of brain extract was incubated at 37°C in the presence or absence of 25 μg granzyme B (purified from human lymphocyte granules, Calbiochem-Novabiochem Corp., San Diego, Calif., USA), in a final volume of 30 μl . At each time point, aliquots were collected and mixed with equal volume of $2\times$ SDS gel loading buffer (100 mM Tris-HCl, pH 6.8, containing 4% SDS, 200 mM DTT, 0.2% bromophenol blue and 20% glycerol) to terminate the reaction. Proteins were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunodetection of caspase-3. Allowing for the possibility that caspase-3 was located in the particulate instead of the cytosolic fraction, in samples devoid of caspase-3 immunoreactivity, total proteins were re-extracted in a harsh lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 4 M urea, 10% glycerol, 5% (v/v) 2-mercaptoethanol) and were analyzed as mentioned above. For caspase-3 and β -actin immunoblot assays, reaction mixtures or brain extracts containing 20 μg total protein were subjected to 12% SDS-PAGE at 100 V for 3 h, and then transferred onto nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad). Blots were washed briefly in PBST (100 mM PB, pH 7.5, containing 150 mM NaCl and 0.05% Tween-20) and blocked with 5% non-fat dry milk in PBST for 1 h. They were then incubated with primary antibody (H-277 rabbit polyclonal anti-caspase-3 antibody, Santa Cruz Biotechnology, Santa Cruz, Calif., USA) diluted in blocking solution (1:2,000) on a platform shaker (12 h, room temperature). After four washes in PBST totaling 30 min, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (HRP-conjugated anti-rabbit antibody, Santa Cruz Biotechnology) diluted in block-

Fig. 1. Expression of caspase-3 in adult (4-year-old) monkey brain in the absence (control) or presence of granzyme B. Brain tissue extracts from the frontal cortex were incubated at 37°C for 0, 5, 15, 30 and 60 min and were then analyzed by Western blotting with a polyclonal anti-caspase-3 antibody (H277). Control, lines 1–5 (incubation in absence of granzyme B): typical immunoblot of brain cytosolic protein extracts showing presence of only one 32-kDa band. Lines 6–9: after incubation with granzyme B, the 32-kDa band gradually vanishes, and a 17-kDa as well as a 20-kDa band appear instead, indicating cleavage of procaspase-3 into its subunits. The H277 antibody therefore recognizes both the inactive and the active forms of caspase-3, but only inactive procaspase-3 is detected in untreated monkey brain extracts.

ing solution (1:2,000, 1 h, room temperature), followed by a thorough wash for at least 30 min with several changes of the buffer. The probed membranes were then incubated with an enhanced chemiluminescence detection reagent (ECL-plus kit, Amersham Biosciences, Hong Kong, China) for 5 min prior to exposure to ECL Hyperfilm (Amersham Biosciences). To control for variations in protein loading, the immunoblots were stripped with Re-Blot Plus strong antibody stripping solution (Chemicon International, Calif., USA) and then probed with monoclonal mouse anti- β -actin primary antibody (clone AC-15, Sigma, St. Louis, Mo., USA; 1:5,000 dilution) followed by HRP-conjugated goat anti-mouse secondary antibody (BD Biosciences, San Jose, Calif., USA; 1:1,000 dilution) and enhanced chemiluminescence detection method. The optical densities of caspase-3- and β -actin-immunoreactive bands were determined with MultiImage II light cabinet (DE-500) equipped with Fluochem Beta 1.1 software (Alpha Innotech, Corp, Calif., USA).

Immunohistochemistry

For immunohistochemical staining of caspase-3 on free-floating sections, fixed brain blocks were transferred to 20% sucrose in 0.1 M PB at 4°C overnight. From these cryoprotected specimens, 25- μ m cryostat sections were cut in the coronal (for neocortex) or sagittal (for hippocampus and cerebellum) plane. Five arbitrary sections from each brain region of individual animals were briefly washed in 0.01 M PB in 24-well plates and incubated in 3% H_2O_2 /10% methanol/0.01 M phosphate-buffered saline (PBS, pH 7.4) for 5 min to quench the endogenous peroxidase, followed by blocking in 5% normal goat serum in 0.01 M PBS. The sections were then incubated overnight at room temperature with the same antibody as used in immunoblot analysis (1:1,000 dilution). After a thorough wash in PBS, caspase-3 immunoreactivity was detected by using Vectastain ABC Elite kit (Vector Laboratories, Burlingame, Calif., USA) according to the manufacturer's instructions with final nickel ion enhancement. In control sections, normal rabbit serum was used instead of the caspase-3 antibody. Additional sections were stained with cresyl violet.

For the quantitative assessment of the proportion of caspase-3-immunoreactive Purkinje cells, sections were examined under a light microscope at 200 $\times$ magnification. Intensely positive, weakly positive and negatively stained neuronal cell bodies were distinguished. Caspase-3-negative Purkinje cells were identified by phase contrast under a relatively darker field in combination with mild focus adjustment. For each monkey (young: $n = 4$; old: $n = 4$), all Purkinje cells in 30 randomly selected fields from five sections were counted and the number of immunoreactive cells was assessed. In addition, the percentage of intensely positive, weakly positive and negative Purkinje cells was determined.

Statistics

Data are reported as mean $\pm$ SEM. For statistical analysis of immunoblot data and of the proportion of immunoreactive Purkinje cells, two-way analysis of variance (ANOVA) was applied, followed by one-way ANOVA and post-hoc Bonferroni tests, where appropriate. A p value <0.05 was considered to be statistically significant.

Results

Specificity of the H-277 anti-caspase-3 antibody was assessed by Western blot analysis of brain extracts from the frontal cortex of 4-year-old rhesus monkeys (fig. 1). In untreated brain extracts (fig. 1, control), only one band was detected corresponding to a molecular weight of 32 kDa, representing inactive procaspase-3. After incubation of brain tissue with 25 μ g granzyme B for 5 min (fig. 1, granzyme B, 5 min), two other bands (17 and 20 kDa) appeared in addition to the 32-kDa band. After longer incubation periods, the 32-kDa band gradually disappeared, and the 17-kDa as well as the 20-kDa bands

Fig. 2. Caspase-3 protein expression in three different brain regions of young (4-year-old, $n = 4$) and old (20-year-old, $n = 4$) rhesus macaque monkeys. **a** Typical Western blots of cytosolic protein extracts showing the presence of a 32-kDa band in all brain regions examined of both young and old monkeys. **b** Densitometric analysis of procaspase-3 protein levels (sample size: 20 μ g protein of brain extract). Data are expressed in relation to the density of β -actin (relative density units). Columns represent means $\pm$ SEM from 4 monkeys each; no significant difference between regions or between age groups is apparent.

became more prominent (fig. 1, granzyme B, 15–60 min). In the presence of human granzyme B, inactive macaque brain procaspase-3 with a molecular weight of 32 kDa was therefore efficiently cleaved into the activated caspase-3 subunits with a molecular weight of 17 and 20 kDa. The 20-kDa peptide is the intermediate product during caspase-3 activation and contains the 17-kDa peptide sequence with an additional pre-peptide at its N-terminus. These data also demonstrate that the H-277 anti-caspase-3 antibody reacts both with the inactive procaspase-3 (32 kDa) and its cleaved active subunits (17 and 20 kDa), but not with 12-kDa small subunit (data not shown). Western blot analysis of different brain regions of 4-year-old and 20-year-old rhesus monkeys (fig. 2) showed that,

both in the young adult and in the aging macaque brain, only inactive procaspase-3 with a molecular weight of 32 kDa was present, but not the cleaved active 17- and 20-kDa fragments (fig. 2a). Moreover, no statistically significant quantitative difference in procaspase-3 expression between 4- and 20-year-old animals was observed in the three regions examined, as assessed by densitometric analysis (fig. 2b).

Immunohistochemical visualization of caspase-3 expression in the brains of young (4-year-old) monkeys revealed distinct regional differences in neuronal caspase-3 distribution (fig. 3–5). In the motor cortex (fig. 3), pyramidal cells of layers III and V showed a moderate immunoreaction of cell bodies, primary dendrites and axons

Fig. 3. Immunohistochemical detection of caspase-3 in the cerebral motor cortex (area 4) of 4-year-old (**a, c, d, f**) and 20-year-old (**b, e, g**) rhesus monkeys. **a, c, f** In the neocortex of a young (4-year-old) monkey, pyramidal cells (arrows), mainly in layer V (**c**), are moderately immunoreactive, whereas small interneurons (**f**, arrowhead) are not labeled. **d** In the white matter, glial cells (arrowheads) are stained, most probably representing astrocytes. **b, e** Note appearance of strongly stained large pyramidal cells (arrow) in aging brain, which are not present in young animals (**a**). These strongly caspase-3-immunoreactive neurons (**e**) show label of cell body and processes. Omission of the primary antibody in control section (**g**) results in negative staining. Scale bars: **a, b** 100 μm ; **c-g** 25 μm .

(fig. 3a, c). The other neocortical layers only displayed a very weak somatic label of neurons (fig. 3f). In the white matter, axons were occasionally weakly immunoreactive. In addition, scattered weakly stained cells with thin branching processes were observed, most likely representing astrocytes (fig. 3d).

In the hippocampus (fig. 4), the most prominent immunoreaction was detected in the somata and processes of hilar neurons (fig. 4a, b). Granule cells of the dentate gyrus were moderately to strongly labeled (fig. 4a, b). Py-

ramidal cells of the CA3 region showed very strong staining of cell bodies, dendrites and axons (fig. 4a, e). Whereas pyramidal cells in CA1 (fig. 4a, c) were moderately to strongly labeled, those in the CA2 region were only weakly immunoreactive (fig. 4a, d). In the molecular layer of CA1-CA3, neurons were generally unstained, only occasionally, cells with a very faint staining were observed.

In the cerebellar cortex, Purkinje cells displayed the most prominent immunoreaction (fig. 5). About 26% of the Purkinje cells showed a moderate to strong somatic

Fig. 4. Caspase-3 immunoreaction in the hippocampus of a young (4-year-old) rhesus monkey. **a** Low magnification, note stronger label in hilar neurons of the dentate gyrus (DG) and CA3 region, as compared to CA1 and CA2 regions. **b–e** Higher magnification of dentate gyrus (**b**), CA1 (**c**), CA2 (**d**) and CA3 (**e**) regions of hippocampus. Note the prominent label of hilar neurons (arrowheads) in the dentate gyrus and of pyramidal cells in CA1/CA3 (arrows). Scale bars: **a** 250 μ m; **b–e** 50 μ m.

and dendritic label (fig. 5b, table 1), often occurring in clusters of 2–3 cells. Other Purkinje cells were only weakly immunoreactive and about 38% were completely unstained (table 1). In the cerebellar granular layer, most cells were unlabeled, only very few scattered granule cells

were weakly stained. In the cerebellar white matter, cells with fine processes displaying astrocyte morphology were moderately labeled.

Caspase-3 immunoreactivity in the brains of aging (20-year-old) monkeys displayed the same overall pat-

Fig. 5. Caspase-3 immunoreaction in the cerebellum of a young (4-year-old) rhesus monkey. **a** Low magnification, note label of some, but not all Purkinje cells. **b, c** Higher magnification: strongly labeled Purkinje cells are often occurring in clusters of 2–3 cells (**c**). Scale bars: **a** 300 μm ; **b** 50 μm ; **c** 25 μm .

tern as that of younger animals, i.e. moderate to strong staining of pyramidal cells in neocortical layers III and V (fig. 3b), of hilar neurons, of pyramidal cells in hippocampal CA1 and CA3 regions, of Purkinje cells and of astrocytes, as well as weak label of most other neurons. Density of immunoreactive Purkinje cells was not significantly different from that of younger animals and there was no significant difference in the percentage of intensely labeled, weakly labeled and unstained Purkinje cells (table 1). A distinct structural difference was, however, observed in the neocortical layer V, where scattered very strongly labeled pyramidal cells were regularly detected in aging (20-year-old) monkeys (fig. 3e), which were not observed in younger (4-year-old) animals (fig. 3c).

In the brains of both 4- and in 20-year-old macaques, there was no morphological indication of apoptotic cell death, i.e., nuclear condensation with accumulation of densely stained chromatin, cell shrinkage, chromatin fragmentation and apoptotic bodies, as visualized in cresyl violet-stained sections (data not shown).

Table 1. Percentage of caspase-3-immunoreactive Purkinje cells in the cerebellar cortex of young (4-year-old) and aging (20-year-old) macaque brains. There is no significant difference between 4- and 20-year-old animals

Age	Purkinje cell-count, %		
	intensely stained	weakly stained	unstained
4 years	25.94 $\pm$ 1.28	36.28 $\pm$ 3.14	37.79 $\pm$ 3.41
20 years	28.03 $\pm$ 2.19	34.09 $\pm$ 3.58	37.88 $\pm$ 2.69

Discussion

The present study determines age-related changes in the cellular localization of caspase-3 in macaque brains. The H-277 rabbit polyclonal anti-caspase-3 antibody utilized reacts only with three clearly delimited bands in the Western blot: one at 32 kDa, the molecular weight of inactive procaspase-3 [13, 23], detected in untreated monkey brain homogenate, and two additional bands at 17 and 20 kDa, representing activated caspase-3 [13, 23, 24],

which appear after exposure to granzyme B, the most powerful proapoptotic granzyme [23, 25, 26]. Human cell culture-derived granzyme B therefore effectively activates procaspase-3 in the macaque brain, despite differences in substrate specificity described between species [26]. Although the possibility of non-specific binding could not be completely ruled out when the H-277 antibody was used for immunohistochemical purpose, high specificity of the anti-caspase-3 antibody was strongly indicated by its successful detection of the full-length procaspase-3 and two derivants of relatively large size (20 and 17 kDa), as well as its failure to react with 12-kDa small subunit in granzyme-B treated monkey brain extract. Blocking of immunostaining by preabsorption with the corresponding antigen theoretically could further strengthen our observations. However, this antibody would require applying the full-length (277 amino acids) antigen, which is not available commercially up to now for this particular use. On the other hand, the antibody we used has already been successfully employed for caspase-3 detection in rodents and humans [27, 28].

In the CNS of young macaques, procaspase-3 immunoreaction is seen in neurons and glia, most probably astrocytes given their morphology and localization. It has been shown that rhesus monkey astrocytes can undergo apoptosis [29, 30], and there is ample evidence that apoptosis of astrocytes is involved in human neurodegenerative diseases [31, 32]. In the rodent brain, procaspase-3 expression and apoptosis of astrocytes have been extensively documented [33–37] and the limitations of the use of GFAP as cell-type marker for apoptotic astrocytes have been discussed in detail, particularly because GFAP-negative astrocytes are most vulnerable to injury [36].

Label is mainly observed in larger neocortical, hippocampal and cerebellar projection neurons, i.e. pyramidal and Purkinje cells, most probably corresponding to inactive procaspase-3, because only the 32-kDa band is observed in the Western blot. Both cell bodies and dendrites are immunoreactive. Procaspase-3 in dendrites and synaptic terminals plays an important role in the local activation of the apoptotic cascade, spreading from the dendrite to the cell body, which has been impressively documented in postischemic brain lesion [38]. Also regulating synaptic plasticity, reversible activation of the apoptotic cascade can occur in synapses during physiological activation of glutamate receptors [38]. Caspase-3, activated in postsynaptic regions of dendrites, can cleave subunits of the AMPA glutamate receptor, thereby decreasing responsiveness to glutamate [39, 40], which may be involved in synaptic plasticity.

Different cell types display different staining intensities. In the hippocampus, the most prominent immunoreaction is observed in hilar neurons and in pyramidal cells of the CA3 region, followed by CA1, whereas CA2 pyramidal neurons are only weakly labeled. This indicates a higher susceptibility to apoptosis of hilar, CA3 and CA1 neurons. In fact, after kainate administration to rats, extensive neuronal cell loss and increased caspase-3 expression are observed in these three hippocampal regions, but not in CA2 and dentate granule cells [41–43]. Hilar neurons are most affected in mesial temporal sclerosis developing after prolonged temporal lobe seizures, sparing CA2 neurons [44–46]. Pilocarpine-induced seizures cause massive apoptosis throughout the entire CA1 and CA3 regions, but neurons in CA2 are most resistant [47]. Increased vulnerability of CA3 pyramidal cells is also observed after traumatic brain injury [48]. Procaspase-3 expression, however, only partly determines susceptibility to injury, because following transient ischemia, CA1 neurons rapidly die, whereas the CA3 region is spared, which has been explained by differential distribution of NMDA receptors and altered potassium currents [38, 49, 50].

The labeling pattern of aging brains is similar to that of young animals. However, scattered very strongly immunoreactive pyramidal cells appear in the aging neocortex, which are not observed in young monkeys. These intensely labeled neurons are not likely to represent dying cells because our brain sections do not show any morphological signs of apoptosis, e.g. chromatin condensation and cell shrinkage [51, 52]. They more probably represent neurons that have become more susceptible to apoptotic stimuli. Increased vulnerability of a subset of neurons occurs in the aging brain, particularly during the development of neurodegenerative disorders [14]. Large pyramidal neurons of layers III and V furnishing long corticocortical projections are considered particularly susceptible to apoptotic stimuli in Alzheimer disease [53]. Interestingly, in the baboon hippocampus, aging is not only associated with upregulation of the proapoptotic protein Bax, but also with redistribution of cytochrome c, which is neither accompanied by caspase-3 activation nor by apoptosis [54].

Accumulation of procaspase-3 in scattered pyramidal cells may also be one of the underlying causes for the increased cytochrome-c-dependent caspase-3 activation observed in the aging macaque brain [22]. There is indication that clustering of inactive procaspases is sufficient for substrate proteolysis [55]. Altered cellular distribution of caspase-3, but also of cytochrome-c [54], may

therefore contribute to age-related changes in caspase-3 activation. These scattered neocortical neurons containing high amounts of procaspase-3 add a further candidate to explain increased susceptibility to apoptosis of the aging brain.

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