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Brain Research Bulletin 74 (2007) 329–335

BRAIN RESEARCH BULLETIN

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Differential response of apoptosis-regulatory Bcl-2 and Bax proteins to an inflammatory challenge in the cerebral cortex and hippocampus of aging mice

Yuan-Zhong Xu [a,b], Xiao-Hua Deng [a,b], Marina Bentivoglio [a,*]

[a] *Department of Morphological and Biomedical Sciences, University of Verona, Verona, Italy*
[b] *Department of Anatomy and Neurobiology, Xiangya School of Medicine, Central South University, Changsha, People's Republic of China*

Received 27 May 2007; received in revised form 1 July 2007; accepted 2 July 2007
Available online 26 July 2007

Abstract

Apoptosis plays a key role in normal aging and neurodegeneration. It is now known that normal aging implies low-grade inflammation and increases susceptibility to neurodegenerative diseases, which, in turn, include a neuroinflammatory component. We here investigated, using mice of 2–3 months, 10–11 months, or 18–21 months of age, the expression of apoptosis-regulatory proteins in cortical brain regions in response to intracerebroventricular administration of pro-inflammatory cytokines. A mixture of interferon-γ and tumor necrosis factor-α was injected, using vehicle (phosphate-buffered saline) as control. At 4 days, levels of the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in the cerebral cortex and hippocampus, examined with Western blotting, were markedly upregulated by cytokine exposure in mice of all age groups with respect to controls. Interestingly, cytokine-elicited Bcl-2 upregulation was aging-dependent, with significant enhancement paralleling the animals' age. Cytokine-elicited Bax expression did not exhibit instead significant aging-related variation. Using the same paradigm and 1 or 2 day survival, Bcl-2 immunoreactivity was observed mainly in neurons of cortex and hippocampus of both control and cytokine-treated mice of all age groups. Furthermore, immunohistochemistry confirmed the enhancement of cytokine-elicited Bcl-2 expression in the cerebral cortex and hippocampus of old mice, and showed that this finding was already evident in the second day after cytokine exposure. The data point out the novel finding that Bcl-2 and Bax expression in cortical brain regions is differentially regulated during senescence in response to an acute inflammatory challenge. Aging-related Bcl-2 increases in neurons after cytokine exposure could contribute to amplify neuroprotective mechanisms in the old brain.

Keywords: Brain; Neurodegeneration; Cell death; Senescence; Cytokines; Neuroinflammation

1. Introduction

A wealth of data indicated in the last years that the normal aging process brings about a low-grade chronic inflammatory condition at the cellular and molecular levels, with increased levels of pro-inflammatory cytokines at the periphery and in the brain, though anti-inflammatory cytokines may also be elevated [7,42]. On the other hand, it is well known that aging is the only established risk factor for neurodegenerative diseases, such as Alzheimer's disease, that are accompanied by a neuroinflammatory component in which glial cells are main players [5,6,30].

We have previously investigated the activation of glial cells in the mouse brain in response to intracerebroventricular (i.c.v.) administration of the pro-inflammatory cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α, showing also that glia activation, which persists for several days, is enhanced during aging [10,18]. Such acute inflammatory challenge was not found to cause neuronal cell death up to 2 days [10]. On the other hand, chronic inflammation has been repeatedly associated with neurodegeneration [4,5,30,32], and acute inflammation can affect neurodegenerative conditions [3,9,39] (see also [2,33] for review).

* Corresponding author at: Department of Morphological and Biomedical Sciences, Faculty of Medicine, Strada Le Grazie 8, 37134 Verona, Italy. Tel.: +39 045 8027158; fax: +39 045 8027163.
E-mail address: marina.bentivoglio@univr.it (M. Bentivoglio).

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doi:10.1016/j.brainresbull.2007.07.002

Apoptosis is involved in neuronal cell death occurring during normal aging [34] as well as in age-related neurodegenerative diseases [27,28,30,32]. The Bcl-2 family of proteins plays a crucial role in the regulation of apoptosis in the nervous system [1,12]. The Bcl-2 member of this family is a 26 kDa intracellular membrane-associated protein which can inhibit apoptosis by preventing the release of cytochrome *c* from mitochondria. The main pro-apoptotic member Bax can form heterodimers with Bcl-2 or Bcl-x_L, thereby abolishing their protective function [21,35]. The Bax/Bcl-2 balance is one of the critical factors determining whether the cell will undergo apoptosis, and this balance can be altered during aging [3,37].

Extending our experimental investigation on the response of the aging brain to neuroinflammation, we here focused on Bcl-2 and Bax expression, examined with Western blotting, in the same paradigm of i.c.v. administration of a mixture of pro-inflammatory cytokines we adopted previously [10]. Immunohistochemistry was used to analyze the morphology of cell types that expressed Bcl-2 after cytokine exposure.

2. Materials and methods

2.1. Animals

Male mice (C57BL/6J) were obtained from Harlan–Nossan (Milan, Italy) and kept in cages in a strictly controlled vivarium, under a 12-h light:12-h dark cycle, with light on at 07:00 a.m., and food and water *ad libitum*. The experiments were performed in animals of three age groups: 2.3–3.5 months, 10–11 months, and 18–21 months old. These groups are here defined as young, middle-aged and old, respectively, following generally accepted definitions of aging in laboratory rodents [8] (see also [25]). All experimental procedures received institutional approval and authorization of the Italian Ministry of Health, and were in accordance with the local committee for animal care and with the European Communities Council (86/609/EEC) directives.

2.2. Surgical treatment

Following deep anesthesia (chloride hydrate, 340 mg/kg, i.p.), the animals were placed on a stereotaxic instrument with a Kopf 921 mouse adaptor (David Kopf Instruments, Tujunga, CA). A mouse brain atlas [12] was used for reference of stereotaxic coordinates for i.c.v. injections. A mixture of 2 μl of recombinant murine IFN-γ and 2 μl of recombinant murine TNF-α (500 U/μl for each cytokine; both purchased from PeproTech EC, London, UK) was injected into the right lateral cerebral ventricle via a Hamilton microsyringe mounted on the stereotaxic holder. This dose of cytokines is the same as that we used in mice of different age groups in a previous study [10]. Control animals received an equivalent volume of 0.01 M phosphate-buffered saline, pH 7.4 (PBS). All injections were made during daytime (between 9:00 a.m. and 2:00 p.m.); the injections were performed over a 10 min period to minimize fluid reflux.

The mice destined to Western blotting analysis ($n = 3$ saline- or cytokine-injected mice per age group) were allowed to survive 4 days, i.e. the longest time interval we previously adopted to investigate glial cell activation [10,18]. This time point was selected in view of the potential long-term consequences of the inflammatory insult, which, as mentioned above, was not found to elicit cell death in the aging brain up to 2 days [10]. At the time of sacrifice, the mice were decapitated and the brains removed.

The mice destined to immunohistochemistry were allowed to 1 day or 2 days ($n = 3$ saline- or cytokine-injected mice per age group and per time point). Under deep anesthesia as above, these animals were perfused transcardially with PBS followed by freshly prepared 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4).

2.3. Western blottings

For collection of tissue to be processed with Western blotting, the hippocampus and cerebral cortex were rapidly dissected out, and immediately homogenized in Dounce homogenizer with five volume (w/v) of ice-cold lysis buffer: 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate and supplemented with 1 mM dithiothretol (DTT), 3 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0.5 mM phenylmethylsulphonyl fluoride (PMSF) just before use. The homogenates were centrifuged (12,000 × *g*) for 15 min at 4 °C after 30 min ice-incubation, and the supernatant was collected and stored at −80 °C until use.

Protein concentration was determined with Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) by spectrophotometry, and bovine serum albumin (BSA) was used for the standard protein curve. Samples were mixed (1:1) with sample buffer (20% glycerol, 4% SDS, 0.02% bromophenol blue and 0.2 M β-mercaptoethanol in 50 mM Tris–HCl, pH 6.8) and denatured in boiled water for 5 min. Protein lysate per tissue from each animal (20 μg) was separated on 12% SDS-polyacrylamide gels and transferred electrophoretically onto polyvinylidene lifluoride membranes (Amersham Bioscience, Little Chalfont, UK). For signal revelation, the membranes were first incubated with 5% BSA in 0.1 M Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature to block free binding sites, and then probed overnight at 4 °C with mouse monoclonal anti-Bcl-2 antibodies (dilution 1:2000; Biosource International Inc., Camarillo, CA) or anti-Bax antibodies (dilution 1:2000; Sigma, St. Louis, MO, USA) in TBS-T containing 1% BSA. The membranes were then washed thoroughly with TBS-T and incubated for 1 h at room temperature with horseradish peroxidase-conjugated polyclonal rabbit anti-mouse IgGs (dilution 1:2000; Dako, Glostrup, Denmark) in the same buffer. The bound antibodies were then visualized with enhanced chemiluminescence (ECL detection kit; Amersham Bioscience) using Hybond-P films. Mouse monoclonal mouse anti-β-actin antibodies (dilution 1:2000; Sigma) were also probed to ensure loading of equivalent amounts of sampled proteins. Bands representing β-actin, Bcl-2, and Bax were determined to be 42, 26, and 21 kDa, respectively, using Rainbow molecular weight standard protein marker (Amersham Bioscience). As negative control, membranes were incubated with the same protocol described above but omitting the primary antibodies. Samples of each animal were independently separated three times and the films were scanned with a laser scanner. Quantification of the signal intensity was performed with Scion image beta 4.0.2 analysis software. Mean optical density was measured densitometrically and normalized to housekeeping β-actin protein.

The data were evaluated as means (mean relative optical density units per group ± standard error of the mean, S.E.M.). Two-way analysis of variance (ANOVA), performed with SPSS 11.5 software, was used to determine the main effects of age or treatment and the interaction between these two variables, followed by the Bonferroni *post hoc* test for pairwise comparisons. The significance threshold was set at $P < 0.05$.

2.4. Histology and Bcl-2 immunohistochemistry

After perfusion, the brains of mice destined to immunohistochemistry were postfixed for 3 h in the same fixative solution, and then cryoprotected overnight in 30% sucrose in PBS at 4 °C. Each brain was then serially cut in the coronal plane at 40 μm thickness on a freezing microtome, and two adjacent series of one every sixth section were collected. One series was processed for immunohistochemistry and the other was stained with cresyl-violet for cytoarchitectonic control and confirmation of the injection needle trajectory.

For the immunohistochemical procedure, free-flouting sections were soaked in 1% H_2O_2 in PBS for 30 min in order to inactivate endogenous peroxidase activity, and preincubated in a solution of 5% normal goat serum, 0.2% Triton X-100, 0.1% NaN_3 in PBS for 1 h. The sections were then incubated overnight at 4 °C in rabbit polyclonal anti-Bcl-2 antiserum (Santa Cruz Laboratories, Santa Cruz, CA), diluted 1:100 in PBS containing 1% normal goat serum, 0.2% Triton X-100, and 0.1% NaN_3. Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgGs (1:200; Vector, Burlingame, CA, USA) for 2 h at room temperature. Finally, the sections were processed with the avidin-peroxidase protocols (ABC, Vector) using 3,3′-diaminobenzidine as chromogen.

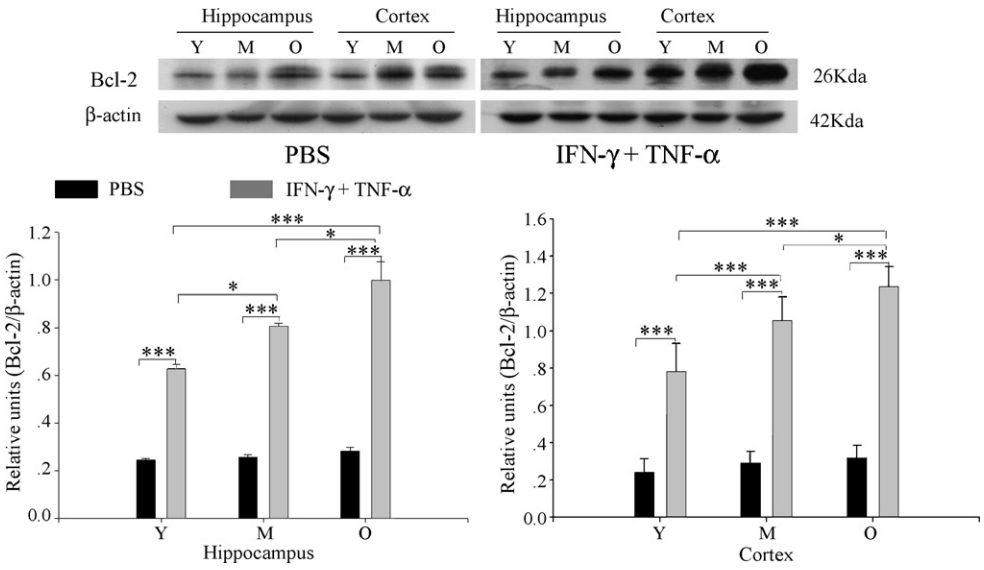

Fig. 1. Western blot analysis of Bcl-2 expression in the hippocampus and cerebral cortex derived from young (Y), middle-aged (M) and old (O) mice after intracerebroventricular injections of vehicle (phosphate-buffered saline, PBS) or a mixture of interferon (IFN)-γ and tumor necrosis factor (TNF)-α. Blots were digitized, and the relative units of optical density obtained by densitometric evaluation, shown in the bar graphs, were normalized to β-actin (data are expressed as mean ± standard error of the mean). The asterisks indicate statistical significance of pairwise comparisons (Bonferroni *post hoc* test following ANOVA): $^{*}P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.0001$.

Control sections were processed with the same protocols but omitting the primary antibody; no specific staining was seen in these sections.

3. Results

3.1. Western blotting

As shown in Fig. 1, Bcl-2 was constitutively expressed in the hippocampus and cerebral cortex of vehicle-treated animals. Although, a slight upregulation was observed in the samples derived from the oldest age group with respect to younger ones, no inter-group significant difference was found in Bcl-2 expression after vehicle administration.

Following the injections of combined cytokines, Bcl-2 expression increased markedly in both the analyzed brain regions (Fig. 1). Interestingly, this upregulation was especially high in the middle-aged and old animals. Two-way ANOVA analyses demonstrated highly significant effects of age and treatment and significant interaction between these two variables in both the cortex and hippocampus (Table 1). Bcl-2 levels in both regions were highest in the cytokine-treated old mice, with significant increases with respect to both the middle-aged and young animals ($P < 0.05$ and $P < 0.0001$, respectively; *post hoc* test following ANOVA). Bcl-2 levels in middle-aged mice also showed a significant increase with respect to young subjects after cytokine treatment ($P < 0.05$ and $P < 0.0001$ in the hippocampus and cortex, respectively; *post hoc* test following ANOVA).

A slight age-related increase was also evident in the study of Bax expression in the hippocampus and cortex of vehicle-treated mice (Fig. 2). However, as observed for Bcl-2, this trend did not show age-related significant differences in control animals (Fig. 2). In all age groups, Bax expression in the two sampled brain regions was markedly upregulated after the cytokine injections (Fig. 2). Two-way ANOVA analyses showed highly significant effects of treatment in both regions and a significant effect of age in the hippocampus (Table 1). Pairwise *post hoc* comparisons confirmed a significant upregulation of Bax levels in all cytokine-treated groups with respect to age-matched controls. However, at variance with the findings observed with Bcl-2, Bax levels did not show significant difference between the three age groups when the interaction of treatment and age in response to IFN-γ and TNF-α exposure was evaluated.

Table 1
Two-way ANOVA statistical analysis performed on Western blotting data

Region	Protein	Main effect of treatment		Main effect of age		Treatment × age interaction	
		$F(1,17)$	*P* value	$F(2,16)$	*P* value	$F(2,16)$	*P* value
Cortex	Bcl-2	765.83	<.0001	32.16	<.0001	18.87	<.0001
	Bax	158.93	<.0001	2.98	Ns	.192	Ns
Hippocampus	Bcl-2	382.42	<.0001	17.46	<.0001	11.69	<.0001
	Bax	62.11	<.0001	5.68	<05	.67	Ns

Abbreviation: ns, not significant.

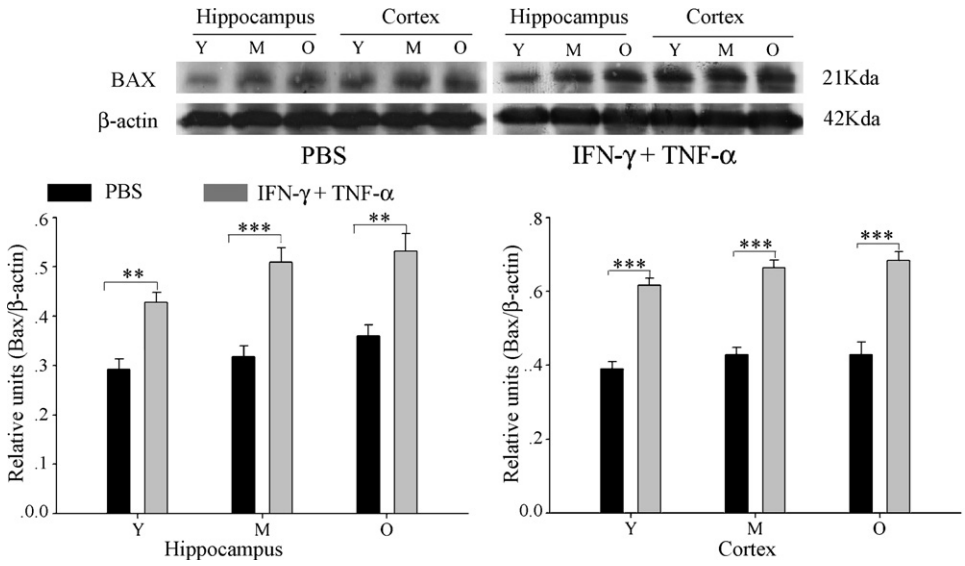

Fig. 2. Western blot analysis of Bax expression in the hippocampus and cortex derived from young (Y), middle-aged (M) and old (O) control mice injected with phosphate-buffered saline (PBS) or cytokine-treated mice which had received injections of a solution of interferon (IFN)-γ and tumor necrosis factor (TNF)-α. As indicated in the legend to Fig. 1, the bar graphs represent the data obtained in the quantitative analysis and the asterisks indicate statistical significance: $^{**}P < 0.001$ and $^{***}P < 0.0001$, Bonferroni *post hoc* test following ANOVA.

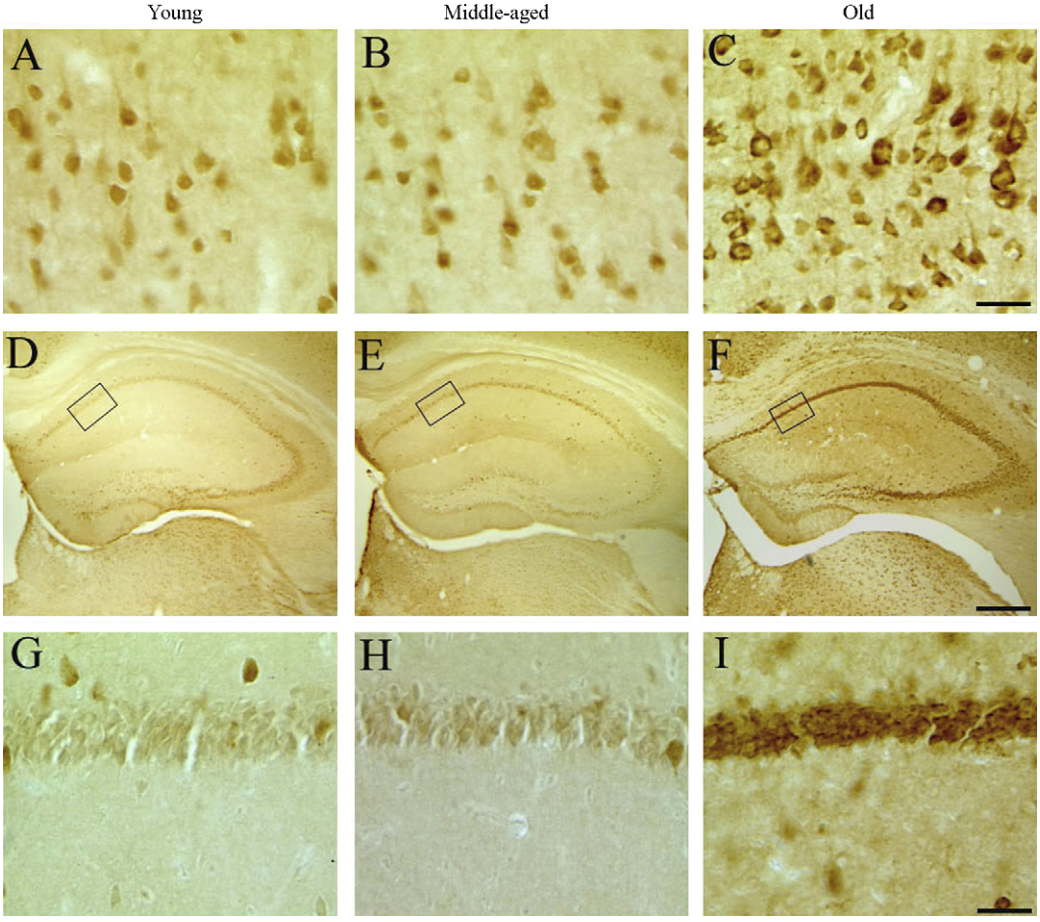

Fig. 3. The plate shows Bcl-2 immunoreactivity in the parietal cortex (A–C) and hippocampus (D–I) derived from young (A, D, G), middle-aged (B, E, H) and old (C, F, I) mice at 2 days after cytokine injections. G, H, I represent at higher magnification the areas boxed in D, E, F, respectively. Note the Bcl-2 immunostaining in neurons (pyramidal cells of the cerebral cortex and hippocampal pyramidal cell layer), and note the enhancement of immunoreactivity in the old mice with respect to younger ones. Scale bars: 20 μm in C and I (applies also to A, B, G, H); 200 μm in F (applies also to D and E).

3.2. Bcl-2 immunohistochemistry

Bcl-2-immunopositive cells were distributed throughout the brain, and were especially numerous in the neocortex, hippocampus and hypothalamic parenchyma surrounding the third ventricle. In all age groups, and in both saline-injected and cytokine-treated brains, the vast majority of these cells were neurons, as clearly shown in Fig. 3 in the cerebral cortex and hippocampus. In the cerebral cortex, Bcl-2-immunopositive cells appeared to be pyramidal neurons, with a distinctly labeled apical dendrite (Fig. 3A–C). In the hippocampus, Bcl-2 immunopositivity was very intense in the CA1 and CA3 fields and in the dentate gyrus (Fig. 3D–F), and was concentrated in the pyramidal cell layer (Fig. 3G–I).

It was well evident that Bcl-2 immunostaining was enhanced after cytokine injection in all age groups with respect to saline injections, especially at 2 days after treatment, although in the old control mice Bcl-2 immunoreactivity was increased with respect to control mice of younger ages. Interestingly, at this time point it was also very obvious that Bcl-2 immunostaining elicited by cytokine exposure was more intense in the cortex and hippocampus of the old mice than of younger ones (Fig. 3). This finding confirmed the age-dependent enhancement of cytokine-elicited Bcl-2 upregulation observed with Western blotting after 4 days survival.

In addition to Bcl-2-immunostained neurons, some elements exhibiting glial features were also seen in the cortex, subcortical white matter and main fiber tracts. These cells were very few and faintly immunolabeled in control saline-injected brains, whereas they were more evident after cytokine treatment. The non-neuronal immunostained elements mostly exhibited the features of oligodendrocytes, and some of them exhibited astrocytic morphology. There were no obvious age-related differences in the Bcl-2 immunoreactivity of non-neuronal cells in both control and cytokine-injected brains. Therefore, the immunohistochemical experiments showed that age-related variation of cytokine-elicited Bcl-2 induction was related to expression of this protein in neurons rather than in glial cells.

4. Discussion

The present data show that the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in cortical brain regions (the hippocampus and cerebral cortex) of mice of different ages are highly upregulated 4 days after co-administration of pro-inflammatory cytokines in the cerebrospinal fluid, and that Bcl-2 upregulation occurs mainly in neurons in both adult and senescent mice. Interestingly, Bcl-2 upregulation in response to cytokine exposure displayed strong age-dependent enhancement, whereas Bax increase did not show significant age-dependent variation. To our knowledge, this is the first demonstration of a differential regulation of the expression of Bcl-2 family members in the rodent brain in response to an inflammatory challenge during adulthood and senescence.

In the current investigation, an aging-related trend towards increased Bcl-2 and Bax expression in the hippocampus and cortex was not found to be significant, in agreement with previous data in rats [38] and in the senescence-accelerated mouse strain [47], although Bcl-2 and Bax upregulation was reported in regions of the aging rat brain in other studies [11,15]. The complex intrinsic pathway of apoptosis implicates an array of molecules and signaling cascades whose investigation is beyond the scope of the present study. It is, however, worth recalling that age-related increase in other markers of apoptotic cell death, including caspase-3 activation, has been reported in the aged rat brain [26]. In non-human primates, the levels of cytochrome *c*-dependent caspase-3 activation were found to be increased in many regions of the brain of old macaques with respect to young ones [48]. Levels of caspase-3 immunoreactivity in different neuronal cell populations of neocortex and hippocampus in old macaques were suggested to account for the selective vulnerability of neuronal subsets in the aging primate brain [49].

On the other hand, the apoptosis pathway displays a complex dependence on species and strains of animals [40]. The investigation of mRNA expression of mitochondrial-encoded genes, including cytochrome *c*, in brain slices from the same strain of mice we used and at the same ages revealed interesting findings [25]. Namely, increased expression of genes in the electron transport chain was found in the specimens from 12- to 18-month-old mice compared to 2 months, with a decrease at 24 months of age, suggesting compensatory mechanisms that cannot be sustained at later stages of aging [25]. In addition, in the study by Manczak et al. [25] high expression of mitochondrial genes was found in the cerebral cortex and hippocampus, pointing to these brain areas as critical for mitochondrial functions. It should also be noted that in the mouse brain an aging-related decline in the intrinsic pathway of apoptosis was reported to be compatible with a decline of apoptotic-activating proteins, including caspase-3 and caspase-9, but was found to be unrelated to inhibitor of apoptosis proteins [40].

As mentioned above, the main findings of our investigation concern, however, the insult elicited by an acute inflammatory challenge. Cytokines tend not to evoke cell death directly when administered individually, but they can exert synergistic effects when co-administered [2]. Amplification of the response to exogenous inflammatory insults has been repeatedly reported in the aging mouse brain [13,14,39,45]. Increased mortality of aged mice in the first 24 h following i.c.v. administration of pro-inflammatory mediators has also been reported [10,14].

Several molecules are involved in the brain response to acute inflammatory challenges, which also implies increases of pro-inflammatory cytokines in the brain of aged mice, as detected after i.c.v. injection of the endotoxin lipopolysaccharide [14]. In the same paradigm adopted in the present study, we have also observed induction of inducible nitric oxide synthase in microglial cells and macrophages of the brain of young mice in the first 2 days after i.c.v. co-administration of IFN-γ and TNF-α [17]. In addition, as mentioned previously, enhancement of microglial activation was found in this paradigm in aged mice [10]. This could, therefore, lead to increased production of the free radical nitric oxide in the aging brain upon cytokine exposure, and previous data have indicated that age-dependent Bcl-2 upregulation in the brain of aged rodents is very sensitive to oxidative stress challenges [15].

Interestingly, the present data add Bcl-2 to the repertoire of genes highly responsive to inflammation in the aging brain, indicating that the Bcl-2/Bax balance in the cerebral cortex and hippocampus may be shifted towards neuroprotection during senescence. Bcl-2 has been shown to rescue various neuronal populations from cell death in a variety of different conditions, including withdrawal of growth factors, axonal damage and ischemia, besides oxidative stress (reviewed in [1,22]). The present data point to an involvement of Bcl-2 in the array of neuroprotective signaling cascades also in the aging brain, which notably implicate neurotrophic factors and suppressors of oxidative stress, as well as other molecules [28].

The present immunohistochemical investigation of the identity of cells expressing Bcl-2 revealed that the vast majority of these elements were neurons, in agreement with previous investigations, in which also Bax expression was found to be primarily neuronal (see, for example, [19,43]). We have also observed Bcl-2 immunoreactivity in some glial cells, as described in previous studies (see, for example, [20,31,43]). However, in our investigation neurons appeared to be the protagonists of changes of cytokine-elicited Bcl-2 expression, including its age-related variation.

Aging and neurodegenerative processes are notably related (e.g. [28]), and apoptosis-regulatory proteins have been repeatedly implicated in the susceptibility of neurons to cell death during senescence. Bax expression was found to be increased in brains affected by neurodegenerative diseases [23,24,29,41,44,45], but other studies reported unchanged Bax expression relative to age-matched normal brains [16,46]. Bcl-2 level was found to be upregulated in the brain of patients with neurodegenerative diseases [16,23,29,32], and it was reported that Bcl-2 increases in parallel with the severity of these conditions [36,41].

The present finding of Bcl-2 enhancement in mice after an acute inflammatory insult during normal aging implicates this protein in adaptive protective mechanisms in cortical regions. The delicate balance between anti- and pro-apoptotic mechanisms and their signaling cascades could, however, be altered by several other factors, including genetic ones, in both acute and chronic inflammatory conditions.

Acknowledgement

This work was supported by grants of the Italian Ministry of University and Research.

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