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PRENATAL IONIZING RADIATION-INDUCED APOPTOSIS OF THE DEVELOPING MURINE BRAIN WITH SPECIAL REFERENCES TO THE EXPRESSION OF SOME PROTEINS

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KEY WORDS

Ionizing radiation; apoptosis; developing brain

ABSTRACT

Apoptosis induced by ionizing irradiation of the developing mouse brain was investigated by using histology, analysis of DNA fragmentation on agarose gel and electron microscopy. A TUNEL-labeled index (L. I.) was calculated from the terminal deoxynucleotidy1 transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay in 4 specific regions, cortical plate, intermediate zone, subependymal zone, and subependymal germinal matrix. The kinetics of apoptosis associated protein was examined by western blotting and immunofluorescence. C57BL/6J mice pregnant on embryonic day 14 (E14) were exposed to a single dose of 1.5-Gy irradiation. Irradiated fetal brains at E15 and E17 showed extensive apoptosis with morphological characteristics. In all 4 regions, L. I. was greater in irradiated brains than in control brains at E15 and E17. Most of TUNEL-labeled cells expressed a mature neuronal marker (NeuN) and Bax protein, which is up-regulated in irradiation-induced apoptosis.

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Ionizing radiation moderately enhanced expression of Bax. Bcl-xL, and Cpp32 proteins. Postnatal irradiated mice showed microencephaly as compared to age-matched mice and the weight of whole body including brain decreased moderately.

INTRODUCTION

The effects of irradiation on the developing mammalian central nervous system (CNS) have been discussed for many years. Many macro- and microscopic analyses have reported that prenatal irradiation of rodent brains induced microencephaly, many dead cells, ectopic gray matter, reduction of cortex thickness, abnormal neuronal migration, and disturbance of synaptogenesis between neurons.[6, 15,16)] Cell death may include both apoptosis and necrosis. In fact, some reports showed that irradiation induced apoptosis in developing brains in association with ATM (Ataxia telangiectasia mutated), p53 or Bcl-2 family proteins, and that these molecules interacted with each other.[1, 6)]

Apoptosis is one of the main features of observed during development of vertebrate brains.[6)] Approximately half of the neurons produced during embryogenesis normally die before adulthood by apoptosis. Because apoptosis appears to occur during synaptogenesis, competition between neurons for a limited amount of target-derived trophic factors may be a primary mechanism of apoptosis, which is associated with the Bcl-2 family.[2, 5)] Widespread expression of Bcl-2, Bcl-xL, and Bax in embryonic neurons of CNS was reported.[4, 19)] Bcl-2 family members that inhibit apoptosis include Bcl-2 and Bcl-xL, whereas Bax constitutes a death-promoting member without a few exceptions.[11, 12)] The hypothesis is generally accepted that the balance between death-promoting and death-repressing members of the Bcl-2 family creates a critical checkpoint that determines whether a cell will execute an apoptotic program which activates the caspase cascade after receiving a death stimulus.[12)]

We examined apoptosis in brains of ionizing-irradiated fetal mouse to elucidate whether there are some differences between the developmental apoptosis and the apoptosis

induced by irradiation and how the apoptosis contributes to developmental disorders.

MATERIALS AND METHODS

Animals and irradiation

Female C57BL/6J mice 6 weeks of age pregnant at embryonic day14 (E14) (Nippon CLEA, Osaka, Japan) were exposed to a single dose of whole-body irradiation at 1.5 Gy (dose rate, l.67 Gy/minute) from a Toshiba X-ray KXC-18 unit (150 kVp, 20 mA) under ambient conditions. Pregnant control mice were sham-treated. Each pregnant mouse was sacrificed by decapitation. Then fetuses were removed from the uterus. Fetuses at E15, E17, and E19 and pups on postnatal day O (PO) r Pl, P3, P5. P7, PIO, P14, and P21 were decapitated and whole brains were removed from the skull for analysis. The brains and whole bodies of postnatal mice were weighed and the brain/body ratio was calculated. Statistical analysis of the brain weight and the brain weight/body weight ratio between irradiated and control mice were performed using Student's t-test.

C57BL/6J mice 6 weeks of age were also exposed to a single dose (3.5Gy) of whole-body irradiation, and decapitated l, 2, 3, 4, 5, and 6 days later, and their whole brains were taken out to compare the susceptibility to irradiation between fetuses and adult mice.

Histology

Brains of all mice were post-fixed in 10% buffered formalin for 7 days, embedded in paraffin, and serially sectioned in the frontal plane at 4-μm thickness. The sections were stained with hematoxylin and eosin (H.E.). KrÜbber - Barrela, and Bodian stains for morphological examination. Analysis by terminal deoxynucleotidy1 transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) method was performed according to the protocol supplied with the in situ apoptosis detection kit, the peroxidase Apop Tag TM assay (Oncor, Inc. Gaithersburg, MD, USA).

TUNEL-labeled index

For E15, E17, E19, and P0 brains, a TUNEL-labeled index (L. I.) was calculated from the TUNEL assay. At least 3 fetuses were examined in each group. In a frontal section including basal ganglia, the cerebral wall (CW) and the subependymal germinal matrix (SGM) were examined. The cerebral walls already consisted of 3 cardinal embryonic zones from the ventricular to the pial surface, namely the subependymal zone (SEZ), the intermediate zone (IM), and the cortical plate (CP). A 100× 100/μm index grid was mounted in an ocular lens and projected onto the tissue sections. A magnification of l000× was used for all analyses. The number of TUNEL-labeled cells and total cells were determined following systematic row sampling for CP, IM, and SEZ, and, following random systematic sampling, for SGM. Briefly, in systematic row sampling, a measuring field (MF) was positioned at the pial surface of the CP, then step by step the MF was moved downwards, each time adjacent to the preceding MF, until it abutted the ventricular surface. The sum of all MFS along the y-axis was a row. In this way, five rows were investigated. In random systematic sampling, the first MF was positioned randomly within the structure, and the next MF was not systematically adjacent to it. Every second MF was considered, giving a total of five MFs. The L.I. (= numbers of TUNEL-labeled cells divided by numbers of total cells) was calculated. The differences in the measured parameters between irradiated and control groups were statistically assessed by F-test, and Student's t-test or Welch's t-test.

Immunohistochemistry

Brains of E15, E17, E19, and PO mice were fixed in 10% buffered formalin, paraffin-embedded and sectioned as described above. In situ protein expressions were examined for immunohistostainings according to the protocol recommended by DAKO LSAB(R) Kit Peroxidase (DAKO Co., CA. USA). Monoclonal antibodies raised against neuronal nuclei (NeuN) (1:100, Chemicon, CA, USA), glial fibrillary acidic

protein (GFAP) (1:100, Santa Cruz Co. , Santa Cruz, CA, USA) . vimentin (Vim) (1:50, IMMUNON, Pittsburgh, USA) , 04 antigen (I : 5 , Chemicon) , and Chk1 (1 : 100 , Santa Cruz Co .) , and polyclonal antibodies raised against Bcl-2 (I : 50, Santa Cruz Co.) . Bcl-xL (1:50, Santa Cruz Co.), Bax (1:100, Santa Cruz Co.), Fas (1:100. Santa Cruz Co.) and Cpp32 (1:100, Santa Cruz Co.) were used as the first antibody.

Immunofluorescence

Brains of E15 and E17 mice were formalin-fixed, paraffin-embedded and sectioned as described above. TUNEL-labeled cells were assessed for immunofluorescence according to the protocol supplied by the Apop Tag R assay kit (Oncor, Inc. Gaitherburg, MD, USA) with a slight modification as described previously.[13] Anti-NeuN, anti-GFAP, anti -Vim, and anti-04 monoclonal antibodies and anti-Bcl-2, anti-Bcl-xL, and anti-Bax polyclonal antibodies were used with the same diluted range as the immunohistochemistry.

Western blotting

Whole brains of E15, E17, E19, and P0 mice were frozen at -80°C. Brain extracts were prepared from whole brains as de scribed previously. [13] The protein concentration was determined by spectrophotometry using the Bio-Rad protein assay (Amersham Pharmacia Biotech, Amesham Place, UK), then l0/1 g of each protein was used for Western blot analysis as described previously.13) For the first antibody, we used anti-Bcl-2, Bcl-xL, Bax, Fas, Ich-1L, Cpp32, and Chk1 antibodies (Santa Cruz Co.) in 0.01 M phosphate buffered saline (PBS) with 0. 1% Tween20 (T-PBS) . For the second antibody, we used horseradish peroxidase-coupled donkey anti-rabbit lgG (Amersham Pharmacia Biotech) diluted to l: 2000 with T-PBS.

Analysis of DNA fragmentation on agarose gel

For analysis of DNA fragmentation, frozen tissues of

whole brains of E15, E17, E19, and P0 mice were added to l ml of DNA extraction solution containing 20mM Tris-HCI (pH 7 . 4) , 0.1 M NaCl , 5 mM EDTA, and 0.5% SDS with 1ml of proteinase K (100µg/ml) , and were minced with a cutter. Tissue lysates were extracted in phenol/tris and then in chloroform: isoamylalcohol (24:1; CIAA). DNA in the aqueous phase was incubated with 5µg/ml DNase-free RNase A at 37°C for I h and extracted with phenol/tris, then CIAA. DNA was collected by precipitation with ethanol. The DNA was dissolved in 50µl TE (l0mM Tris-HCI and 1mM EDTA), separated on 1.8% agarose gel containing 1µg/ml ethidium bromide, and DNA fragments were made visible under UV light.

Electron microscopic analysis

For observation by electron microscopy, brains of E15, E17, E19, and P0 mice were divided into several pieces and immersed in Karnovsky's fixative [4% paraformaldehyde, 5% glutaraldehyde in 0.1 M phosphate buffer (PB)] for 2 h and postfixed with l% osmium tetroxide in PB for 2 h. Tissue blocks were embedded in Epon 812. Ultra-thin sections (60nm) were cut and stained with uranyl acetate and lead citrate. The sections were examined under electron microscopy.

RESULTS

Apoptosis and TUNEL-labeled index

The morphological examinations revealed numerous apoptotic cells characterized by nuclear fragmentation and condensation were observed in the telencephalon of irradiated mice at E15 and E17 with the highest number seen in the IM. These cells were TUNEL-labeled (Fig.1A). A few cells were TUNEL-labeled in the same regions of the control brains (Fig.1B). On the other hand, adult mice irradiated at 6 weeks showed few apoptosis in the brains after I day through 6 days of exposure (data not shown).

Figure 2 shows an analysis of DNA fragmentation at E15. Chromatin fragmentation was visible in the irradiated brains at E15 and E17. The fragmentation was observed more markedly

at E15 than at E17.

By electron microscopy, many cells showing condensed chromatin and cell shrinkage, which are typical features of apoptosis, were found in the irradiated cortex of E15 and E17 brains (Fig. 3). In both irradiated and control groups, the intracellular organelles developed: the number of mitochondria increased according to age. Increases in the number and length of axon were also observed.

Table I shows the TUNEL L.I. In all regions of the brain examined (SEZ, IM, CP, and SGM), the L.I. of the irradiated groups was significantly higher compared with that of the control groups at E15 and E17 (Welch's t-test; $p<0.01$). All L.I. of E15 mice were higher than those of E17 mice. The L.I. of the IM from irradiated E15 brains was the highest among all L.I. examined. In the CP of irradiated P0 mice, L.I. was slightly higher than that in the control (Student's t-test and Welch's t-test; $p<0.05$). In SEZ, IM, SGM of E19 and PO, and CP of E19, no significant differences were recognized between the two groups (Student's t-test and Welch's t-test; $p>0.05$).

Kinetics of the related proteins

Figure 4 shows a TUNEL assay, in combination with immunohistochemical analysis using anti-NeuN, Bax. Bcl-2 and Bcl-xL antibodies in the CW of irradiated and control E15 fetuses. In the CP and IM of the both irradiated and control brains, most TUNEL-labeled cells showed immunoreactivity for NeuN (Fig.4), but few TUNEL-labeled cells showed immunoreactivity for GFAP, Vim, or 04 (data not shown). In the SGM, abundant TUNEL-labeled cells expressed no phenotypic marker of astroglias and oligodendrocytes, and only a few expressed NeuN (data not shown). A scattered expression of Bax, Bcl-2, and Bcl-xL proteins was found in the CP and IM. Most TUNEL-labeled cells showed immunoreactivity for Bax protein in the CW of the irradiated mice. Some showed immunoreactivity for Bcl-xL protein, and a few showed for Bcl-2 (Fig.4). All TUNEL-labeled cells in the SGM expressed Bax protein, and some expressed Bcl-2

protein, but none expressed Bcl-xL protein in the irradiated brains. In the CP, IM, and SGM of the control brains, a few TUNEL-labeled cells expressed both Bax and Bcl-2 protein, but no Bcl-xL protein.

Figure 5 shows expression level of specific proteins examined by western blot analysis. The levels of Bax and Bcl-xL proteins were increased in the irradiated brains compared with those in the control brains at E15, E17, E19, and PO. The level of Bcl-2 protein was much the same in both groups or slightly increased in the control brains compared with that in the irradiated brains. No significant difference of the level of Chkl was found between both groups from E15 through PO. The expression level of Chkl was high at E15 and E17 and decreased at E19 and PO in both groups. The level of Cpp32 was a little higher in the irradiated brains compared with the control brains at E15 and E19 (data not shown). The levels of Fas and Ich-IL proteins were low prenatally, and there was no significant difference between irradiated and control brains at any age (data not shown).

Developmental disorders

The postnatal cerebral cortex was thin, and the size of the whole brain was decreased in the irradiated brains compared with the control brains (Fig.6A. B). The 6 cortical layers were formed and gross malformations were not found in either group at any age.

The weight of the irradiated brains was lower at P7 and p21 than that of age-matched control brains (Student's t-test; $p<0.05$). There was no significant difference in the ratios of brain weight to body weight between irradiated groups and control groups (Student's t-test; $p>0.05$) (data not shown).

DISCUSSION

The extent of damage depends upon the irradiation dose phase-specific vulnerability to and gestational irradiation.6) The present study has revealed that abundant

TUNEL-labeled pyknotic cells were observed in the cerebral cortex of E15 and E17 fetuses which were irradiated at E14. Furthermore, the analysis of DNA fragmentation on agarose gel and observation by electron microscopy confirmed significant apoptosis in the irradiated brains. By contrast, few TUNEL-positive cells were found in the brain of the adult mice I to 6 days after irradiation at the age of 6 weeks. These results show that fetuses were more sensitive to irradiation than adult as consistent with previous report.6)

TUNEL-positive cells ' were prominent in the telencephalon of irradiated mice, especially in the CW and the SGM (Fig.1A). L.I. showed its highest score in the irradiated IM at E15 (Table I). Since at E15 and E17 many young neurons are on the ways of migration from the SEZ to the CP.16) the TUNEL-labeled cells in the irradiated IM at E15 and E17 may be the cells located in the SEZ at E14 when they are irradiated.

Most TUNEL-labeled cells expressed Bax protein, and the protein level of Bax from whole-brain lysates was higher in irradiated mice than that in the normal control, suggesting that Bax is one of the promoters of apoptosis following ionizing irradiation in the telencephalon of mice. The present data agree with the previous report analyzing irradiated fetal rats.1)

Bax-deficient mice are viable, [7, 19)] and there is no difference in developmental apoptosis in the telencephalon between a Bax null-mutant mice and the wild type at Ell.5 through Pl, although widespread elimination of apoptosis occurs in the many regions including the brainstem, cerebellum, and hippocampus.[19)] Bcl-$2^{-/-}$ mice are also viable and their nervous system appears normal. [10, 18)] Bcl-x deficient mice die around E13 and differentiating neurons in the IM of this mutant show extensive apoptosis, in which the bcl-x mRNA is expressed in wild-type mice.[4, 9)]

The most extensive apoptosis was induced by ionizing irradiation in the IM with expressing Bcl-xL protein. Although the number of TUNEL-labeled cells was increased in irradiated brains at E15, anti-apoptotic protein Bcl-xL was up-regulated at E15 through P0, paradoxically. Bcl-xL and

Bcl-2 heterodimerize with Bax, and this heterodimerization is required for the repression of apoptosis.[14] In our study, the protein level was assessed at least a day after irradiation and the occurrence of apoptosis is maximum at that day. The result suggests that the initiation of the apoptosis occurs within 24h after irradiation and subsequently the level of Bcl-xL protein increases to repress the excess apoptosis. It was reviewed that Bcl-2 family proteins have two way s o f controlling apoptosis, by mitochondrial permeability transition (megapore opening) and release of cysteine protease (caspase) activators from mitochondria, and by interacting with other proteins suspected to mediate caspases.12) Our results confirmed that Cpp32, terminal member of caspase cascades, but not Ich-IL, was essential to the apoptosis in developing mouse brains. Fas protein was not involved in apoptosis induced by ionizing radiation in fetus.

Chkl is required for the G21M DNA damage checkpoint.17) $Chkl^{-/-}$ mice is embryonic lethal and in cultured $Chkl^{-/-}$ blastcysts many cells in the inner cell mass show apoptosis. [8] Western blotting analysis in the present study showed that expression of Chkl was clearly detected at E15 and E17 and decreased on E19 and PO both in the control and irradiated brains.

Most TUNEL-labeled cells were immunoreactive for NeuN, which is a specific marker for young neurons. This finding suggests that most cells targeted by apoptosis due to prenatal ionizing irradiation in the mouse brains were neurons, although Sasaki et al. reported that apoptosis targeted neurons and oligodendrocytes in the dentate gyrus of adult mice.[13] Gobbel et al. showed that neurons are more susceptible to apoptosis by x-irradiation than astrocytes. [3]

Much neuronal apoptosis in irradiated mice at E15 and E17 was associated with postnatal thinner cerebral cortex, decrease of the size of the whole brains and a reduction in brain and whole body weight. Further examinations will be necessary to evaluate the precise cooperation of apoptosis-regulating molecules under varied conditions of the developing CNS.

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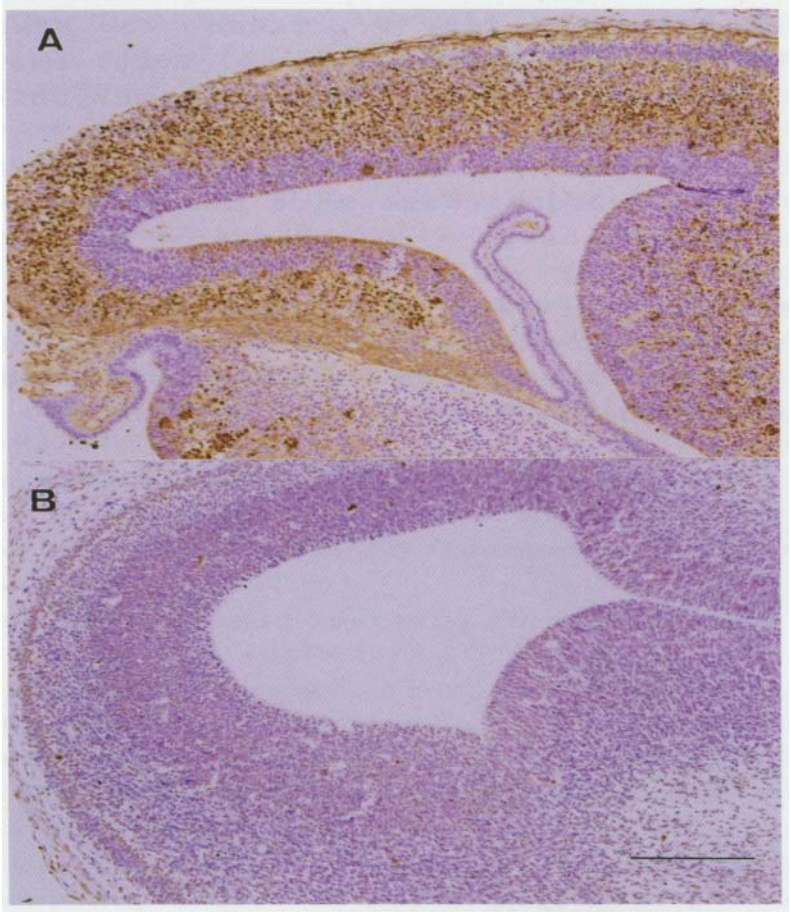

Figure 1 . Abundant TUNEL-labeled cells are seen in the irradiated E15 cerebral wall (CW) and subependymal germinal matrix (SGM) demonstrated by TUNEL-staining and hematoxylin for nuclear staining (A). By contrast, the CW and SGM of control E15 fetus show a few TUNEL-positive cells (B). Scale bar, 200μm.

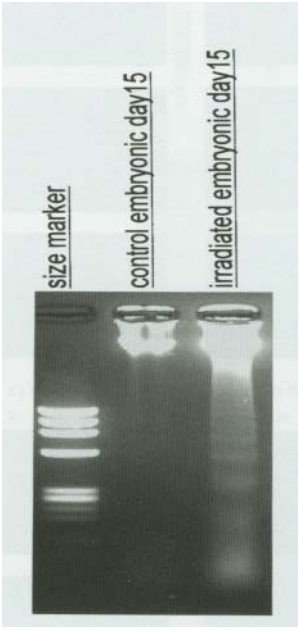

Figure 2. Analysis of DNA fragmentation on agarose gel at E15. Irradiated E15 DNA shows chromatin fragmentation.

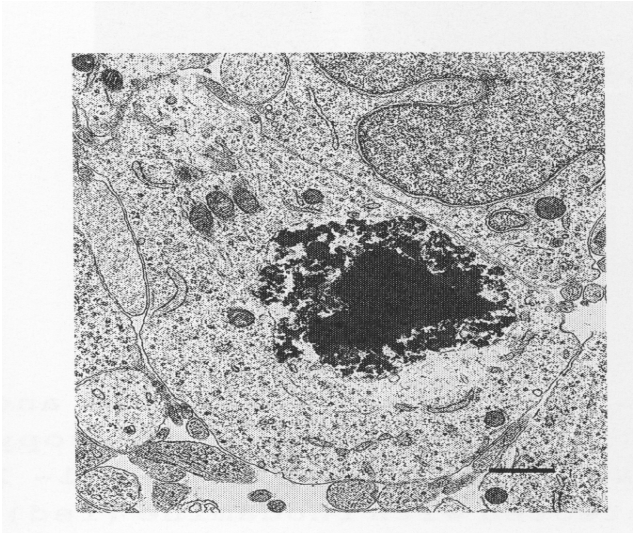

Figure 3. Apoptotic cell showing condensed chromatin and fragmentation and maintenance of the cytoplasmic organelles in the irradiated murine cerebral wall at E15 is demonstrated by electron microscopy. Scale bar. μm.

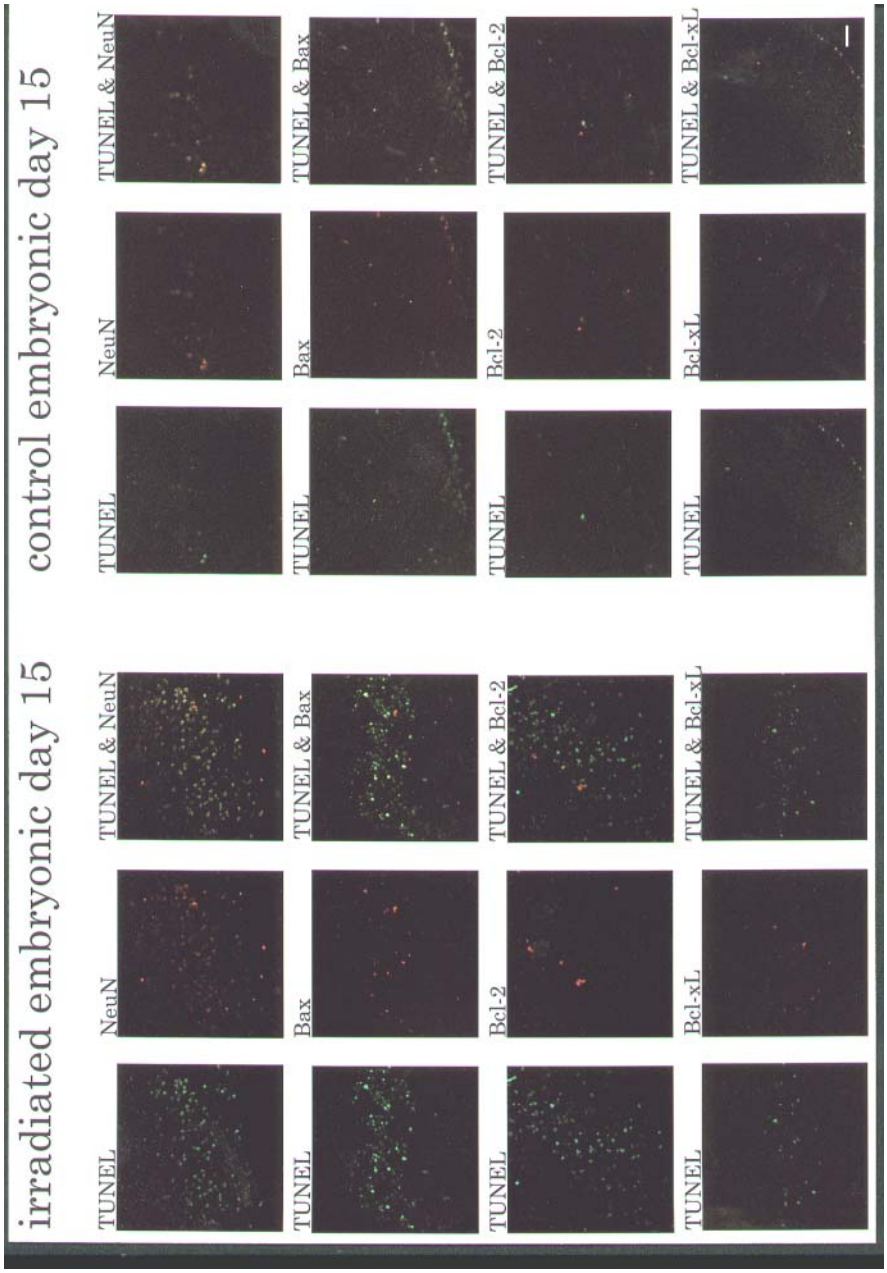

Figure 4. Immunohistochemical analysis of irradiated and control murine cerebral wall at E15 using confocal microscopy. The immunoreactivities of anti-NeuN. anti-Bax. anti-Bcl- 2 and anti-Bcl-xL antibodies are detected with rhodamine (red) . and TUNEL-positive cells are detected by FITC (green). Scale bar. 30/μm

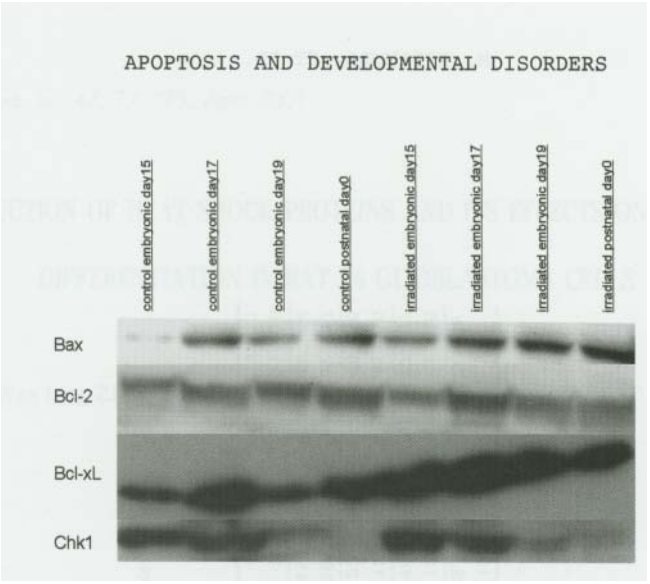

Figure 5 . Western blot analysis of mouse whole brain homogenates using anti-Bax, anti-Bcl-2, anti-Bcl-xL, and anti-Chkl antibodies, respectively. The size of each protein band corresponds to expected size.

Figure 6. Postnatal day 21 whole brain of irradiated (A) and control (B) mice. H.E. staining. Scale bar. 1mm.

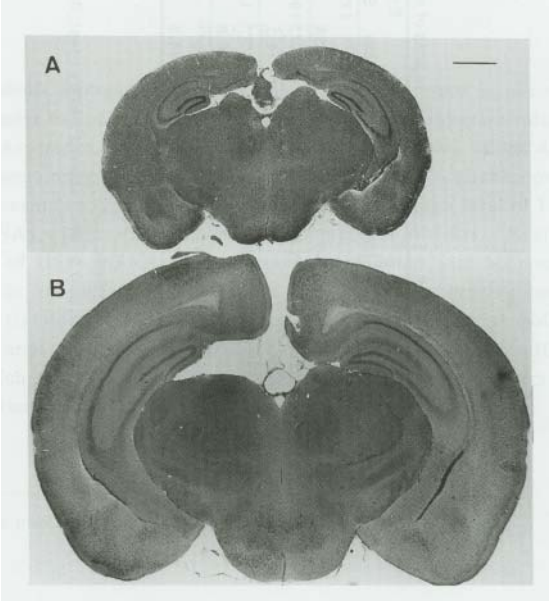

Table I: TUNEL-labeled index (L.I.) in the four specific regions of murine brains.

		E15			E17			E19			P0		
		Mean	S.D.	Co.	Mean	S.D.	Co.	Mean	S.D.	Co.	Mean	S.D.	Co.
Cortical	irradiated	1780**	1380	34	948**	1730	47	84.3	132	75	136*	217	126
Plate	control	0.238	13	30	7.58	38	51	56.4	108	65	75.1	177	87
Intermediate	irradiated	4580**	1240	41	1260**	1920	39	81.1	122	71	449	635	50
Zone	control	21.6	44	38	11.1	36	53	130	238	62	244	468	55
Subependimal	irradiated	1860**	987	34	914**	144	42	52.2	56.8	37	144	212	37
Zone	control	23.6	44	40	18.5	36	39	34.2	41.3	34	103	166	44
Subgerminal	irradiated	2430**	1150	15	1320**	99	15	78.3	84	20	87	101	20
Matrix	control	31.7	35	15	44.3	47	15	52.4	49	15	98.7	88.9	21

Mean, S.D. : $\times 0.0001$

Co. : the count of measuring fields

** revealed Welch's t-test; $p<0.01$ and * showed Welch's t-test; $0.01<p<0.05$, respectively.