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Analysis of Histopathology of the Irradiated Adult Rat Brain During First 3 Post-Irradiation Months : Expression of Protein Kinase C and the Role of Apoptosis in Radiation Encephalopathy

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방사선조사후 3개월동안의 흰쥐뇌의 병리학적 소견 : protein kinase C의 변화와 apoptosis의 역할

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목 적 : 방사선 조사에 의한 뇌의 병변은 임상에서나 실험동물에서 일정기간의 잠복기후에 주로 혈관과 상의세포하층의 세포의 변성에 의해 나타나는 것으로 이해되고 있다. 본 실험은 변화가 주로 일어나는 것으로 알려져 있는 구조들에서 방사선 조사후 초기의 변화를 관찰하여 방사선 뇌병변의 병태생리학적 기전을 밝히는데 도움이 되고자 하여 계획되었다.

방 법 : 성숙 흰쥐의 두부에 20Gy의 방사선을 조사한 다음 10분, 1시간, 3시간, 1일, 3일, 9일, 3개월후에 측뇌실에 있는 상의세포하판의 세포와 맥락총의 혈관과 상피세포, 신경망, 그리고 대뇌피질과 해마신경세포의 변화를 관찰하였다. 면역조직화학검사로 protein kinase C(PKC) gamma와 glial fibrillary acidic protein(GFAP)의 변화를 관찰하였으며 상의세포하판의 변화는 시간에 따른 세포수의 변화와 유사분열의 유무, apoptosis의 빈도를 보았다.

결 과 : 맥락총의 상피세포는 공포성 변화를 보였고 혈관은 3개월에 내강의 확장을 보였으나 다른 변화는 보이지 않았다. 측뇌실 상의하판의 미분화세포에서 가장 현저한 변화가 관찰되었는데 방사선 조사후 10분부터 apoptosis가 보이기 시작하여 3시간에 가장 심했으며 그후로는 세포의 수가 심하게 감소되어 10일에 정상의 10%이하였고 3개월에는 0%로 감소되었다. Apoptosis는 치상회에서도 비교적 자주 관찰되었고 해마의 다른 부위에서도 간혹 관찰되었다. PKC γ 는 맥락총의 상피세포에서는 세포질에서 세포막으로 이동되었고 상의하판의 미분화세포에서는 방사선 조사후 3시간부터 지속적인 증가를 보였다.

결 론 : 방사선 조사후 9일까지의 조기의 변화와 3개월후의 변화를 관찰한 결과 상의하판의 미분화세포에서 apoptosis가 관여하는 가장 심한 변화를 보였으며 PKC γ 의 변화가 보여서 방사선 조사에 의한 성숙 흰쥐뇌의 병변에서 apoptosis와 PKC γ 가 중요한 역할을 하는 것을 알 수 있었다.

Intoduction

The pathogenesis of irradiation induced damage in central nervous system(CNS) has been a great concern and interest clinically because of the effects of radiation therapy of brain tumor on adjacent brain parenchyme. Radiation-induced damages of CNS are mostly early delayed(2~13 weeks after irradiation) or late delayed(months or years after irradiation) types. Early delayed changes consist of demyelination with axonal break down and perivascular infiltration of mononuclear cells. The late delayed effects consist of blood vessel changes such as fibrinoid necrosis of vascular wall with vascular thrombosis and telangiectases, and coagulative necrosis and demyelination of white matter. The mechanism of post-irradiation white matter necrosis has been known to be associated with loss of reproductive ability of cells of the subependymal plate[1)2)] as well as vascular damages[3)4)].

Dose-limiting tissue components in CNS of rats, previousely defined as "Tissue Injury Unit(TIU)", consist of a dilatation of the vascular lumen, a thickening of the blood vessel wall, an enlargement of endothelial cell nuclei, and a hypertrophy of the adjacent astrocytes, which proved to be slightly more sensitive and responsive than the earliest recognizable changes in the neurological structures, demyelination[5)-7)]. Reinhold et al.[7)] showed those parameters contributing to "TIU" present a much stronger correlation with demyelination than with number of astrocytes, oligodendrocytes or endothelial cells. Other studies also showed that a decrease in oligodendrocytes alone is unlikely to cause necrosis and that vascular damage must play an important role[8)9)]. After 20Gy irradiation, no signs of demyelinatin could be detected within period of 1 year.

Protein kinase C(PKC) gene family has been known to play an important role in tumor promotion and regulation of cell growth. Protein phosphorylation by PKC with consequent secretion and degranulation of lysosomal enzymes, and activation of the oxidative burst is critically involved in the function of the nervous system. Protein kinase A, C, and Ca^{2+}/calmodulin-dependent protein kinase II phosphorylate several proteins involved in neurosecretion and cytoskeletal organization. Activation of PKC is expressed in multiple ways : in translocation of PKC from cytosol to the membrane ; increased intracellular concentration of the enzyme ; increased intracellular PKC-specific kinase activity ; and increased expression of PKC mRNA[10)-13)]. Previous experiments showed X-rays can induce increased expression of PKC mRNA within 1 hour after irradiation exposure 4 to 6 folds over unirradiated controls[14)-17)].

This study was planned to see the changes taking place during latent period before delayed radiation necrosis of the CNS. The changes in components of "TIU" as well as subependymal plate(SEP) were evaluated at different times during the first 3 months following 20Gy irradiation of the rat brain. Expression of PKC γ was observed in each components and matched with other changes. In addition, we also present the incidence of apoptosis in different areas of the rat brain and discuss its meaning.

Material and Methods

1. Animals and experimental design

Sprague-Dawley rats of both sexes weighing 200-250 gm were used for the present study. Each rat was allowed free access to food and water before and after the radiation. The rats were lightly anesthetized with ketamine(50mg/ml) intraperitoneal injection(100mg/kg). Whole brain was irradiated 20Gy by 6 MV linear accelerator(NEC 1000x) through anterior 1 portal with shielding of both eyes.

For the evaluation of various postirradiation periods, the rats were followed for 10 minutes, 1 or 3 hours, 1, 3, or 9 days, and 3 months. At least one sham-operated rat(no irradiation took place) as a control was included with each group of four irradiated rats.

2. Tissue preparation and immunohistochemical method

At the scheduled time, each rat was anesthesized and the brain was fixed with transcardiac infusion of 4% paraformaldehyde following perfusion with isotonic saline to remove blood from the cerebral vasculature. The brains were removed and fixed in the same solution for a further 24 hours. Coronal sections of the supratentorial portion of each brain were taken

and embedded in paraffin. Routine sections were stained with hematoxylin-eosin (H-E) and luxol fast blue (LFB) to demonstrate changes of myelin as well as immunohistochemical study with anti-PKC γ and anti-glial fibrillary acidic protein (GFAP) antibodies to evaluate the changes of signal transduction in various cells and reactive changes of astrocytes, respectively.

The immunohistochemical reaction with anti-PKC γ and anti-GFAP antibodies was accomplished using peroxidase-antiperoxidase method as described with some modification[18]. Briefly, each deparaffinized 5-μm coronal section was reacted with a primary antiserum (see below) for 60 min before reaction with the peroxidase-antiperoxidase complex by LSAB kit from DAKO (Santa Babara, CA, USA). The peroxidase reaction was carried by incubation with link antibody and streptavidin for 20 min, respectively, and subsequently with AEC (3-aminoethyl 9-carbasol). The sections were counterstained with Meyer's hematoxylin to visualize cell nuclei. A coronal section was incubated with non-immunized serum from the same species used to raise each primary antiserum. No control section showed a positive reaction. The antiserum for PKC γ from rat brain, raised in Balb/C mice, was purchased from Zymed (San Francisco, CA) and diluted to 1 : 100. The antiserum for GFAP from human brain, raised in mouse, was purchased from Dako (Glostrup, Denmark) and diluted to 1 : 100.

Results

1. Autopsy

The body weight was not changed significantly up to 3 days and reduced 30-60 gms (average 50gms) at 9 days and increased 20~120gms (average 50gms) at 3 months, respectively. External lesions observed were hair loss within irradiation field and crusty deposits around the eyes and the head was tilted to one side. The rats in control group did not show gross anomalies.

2. Histopathology

The histopathological analysis was performed in anatomical regions of interest, which has been known as main target of irradiation in the brain, such as white matter, blood vessels in choroid plexus in lateral ventricles, and subependymal primitive cell layers. The changes of blood vessels and perivascular edema were graded 0 to 3+, where 0 was absence of changes and 1+, 2+, and 3+ were mild, moderate, or marked severity, respectively. PKC and GFAP immunoreactions (IR) were graded 1+ to 3+ as mild, moderate, and marked positive reaction. The subependymal primitive cell layers were evaluted for degree of apoptosis and loss of cells with reactive glial proliferation. Apoptotic changes of cells were graded 0 = normal, 1+ = less than 10% of cells were apoptotic, 2+ = 10~50% of cells were apoptotic, and 3+ = more than 50% of cells were apoptotic. The hippocampi and frontoparietal cerebral cortex were also evaluated for presense of degenerated neurons.

1) White matter changes

All the animals showed well preserved white matter with no evidence of edema, necrosis, or demyelination.

2) Changes in epithelial cells and blood vessels of choroid plexus in lateral ventricles

The epithelial cells showed mild (1+) vacuolation in all 4 rats at 10 min, moderate (2+) vacuolation in 2, 3, and 1 out of 4 rats at 1, 3, and 24 hours, respectively (Fig. 1a-c). At 3 and 10 days, 2 out of 4 rats showed moderate and mild vacuolation of epithelial cells, respectively. At 3 months, all 5 rats showed moderate vacuolation of epithelial cells as well as moderate degree of vascular dilatation (Fig. 1d). Mild to moderate perivascular edema was noted in 1 rat at 1, 3, and 9 days.

PKC γ was finely dispersed in cytoplasm of epithelial cells in normal control rats (F 2a) and marginally displaced after the irradiation with no discernible changes of quantity up to 3 months (Fig. 2b). Ependymal lining cells also showed positive reaction with no time sequential changes.

3) Changes in subependymal plate (SEP)

In normal rats, subependymal plate (SEP) about the rostral end of the lateral ventricle showed many undifferentiated primitive cells with active mitosis (Fig. 3a). PKC γ was negative in SEP in lateral ventricle

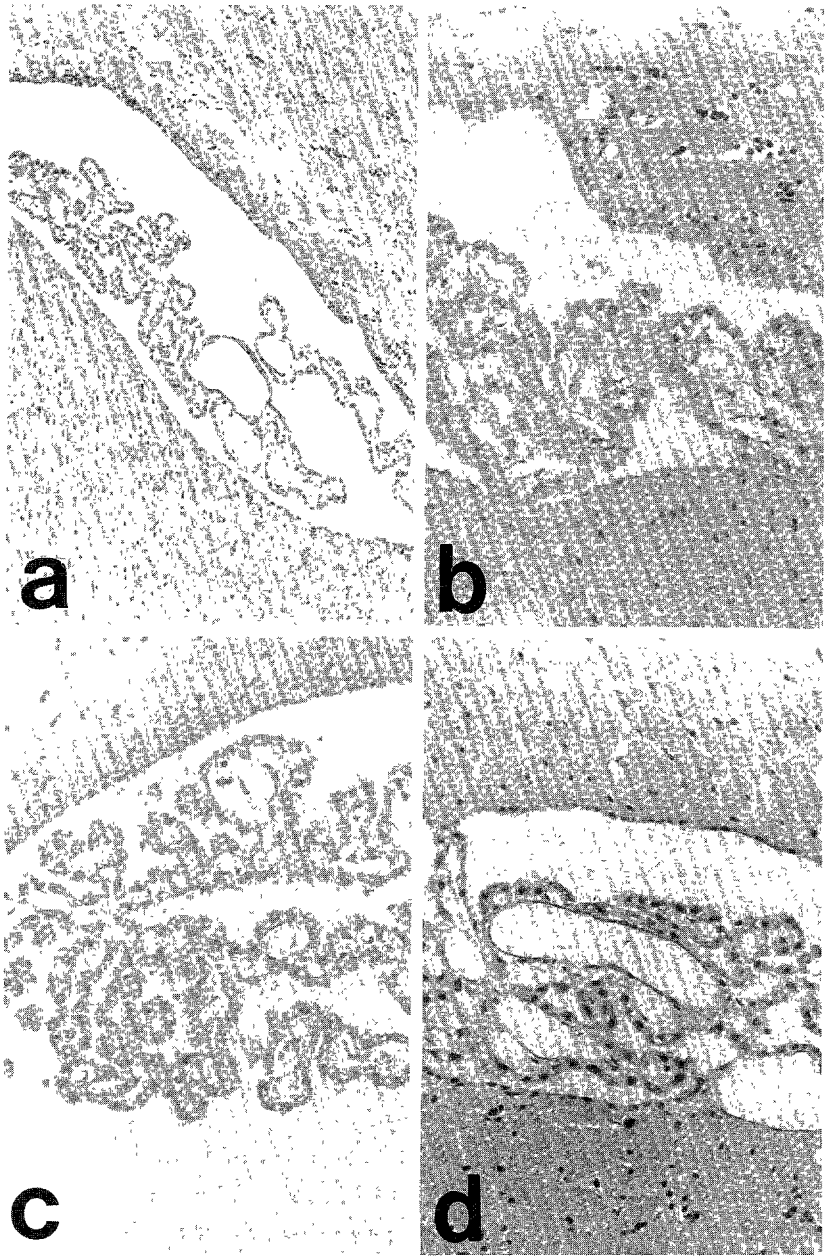

Fig. 1. a : Choroid plexus in normal control rat(H-E, ×20)
b : Mild(1+) vacuolation of epithelial cells of choroid plexus at 3 hours postirradiation(H-E, ×50)
c : Moderate(2+) vacuolation of epithelial cells of choroid plexus at 1 day postirradiation(H-E, ×50)
d : Mild vacuolation of epithelial cells and moderate vascular dilatation at 3 months postirradiation(H-E, ×50)

(Fig. 3b) compared to positive reaction in ependymal lining cells. GFAP showed strong positive reaction in SEP as well as in adjacent ependymal linings(Fig. 3c).

Subependymally located medial habenular nucleus (Mhb) around third ventricle was compared with SEP, which showed more differentiated cells(Fig. 3d). PKC γ

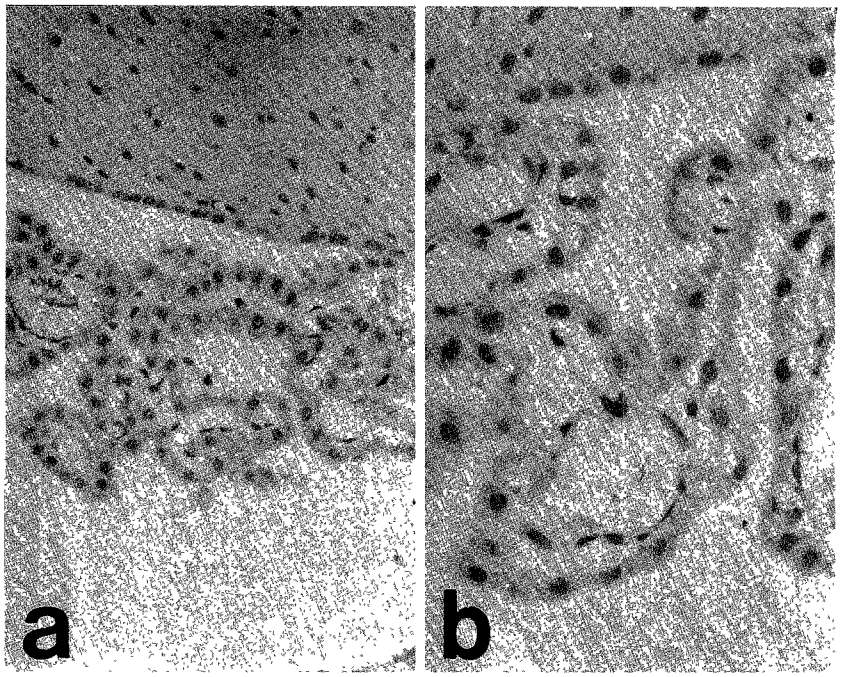

Fig. 2. a : Finely dispersed PKC γ in cytoplasm of epithelial cells in normal control(PAP, ×50)
b : Marginally displaced PKC γ with vacuolation of epithelial cells in 10 min postirradiation(PAP, ×100)

was positive in background(Fig. 3e) and GFAP showed mild positive reaction in Mhb with negative reaction in ependymal linings of third ventricle(Fig. 3f).

At 10 min post-irradiation, SEP showed several apoptotic cells in 3 rats(75%) compared to no discernible change in Mhb. Apoptotic cells were increased in numbers at 1 hour and maximum in numbers at 3 hours in all 4 rats(100% of rats, over 75% of cells)(Fig. 4a) and decreased at 1 and 3 days in 4 and 2 rats, respectively. The numbers of cells were markedly decreased to less than 25% cell density compared to control(Fig. 4b). The cell density in SEP was less than 10% of normal control at 10 days and 0% at 3 months(Fig. 4c & d). The lateral ventricle was markedly dilated at 3 months.

The changes in Mhb were much less and later than SEP, showing only scattered cells with hyperchromatic condensed nucleus at 3 hours, which were slightly increased in numbers throughout 1, 3, and 10 days. Several apoptotic cells were noted in 1 out of 4 rats at 10 days. At 3 months, the cells in Mhb showed scattered pknotic cells with cell density of over 90% of normal control rats.

PKC γ was increased in SEP after 3 hour throughout to 3 month and transiently increased in Mhb at 3 days. GFAP reaction was markedly increased in SEP after 1 day.

4) Changes in neurons of hippocampi and frontoparietal cortex

Neuronal changes were not sequential and quite haphazard. Degenerating neurons with pknotic nuclei were first noted in cortex and hippocampus at 10 min post-irradiation and granular cells of dentate gyrus (DG) showed more changes than those in cornus ammonis(CA). The neurons in DG showed apoptotic cells in 1 rat at 3 hours and those in CA2-3 showed several apoptotic cells in 1 rat at 3 days. The changes were similar at different times.

Discussion

The body weight was considerably decreased (average 50gms) at 9 days after irradiation and slightly

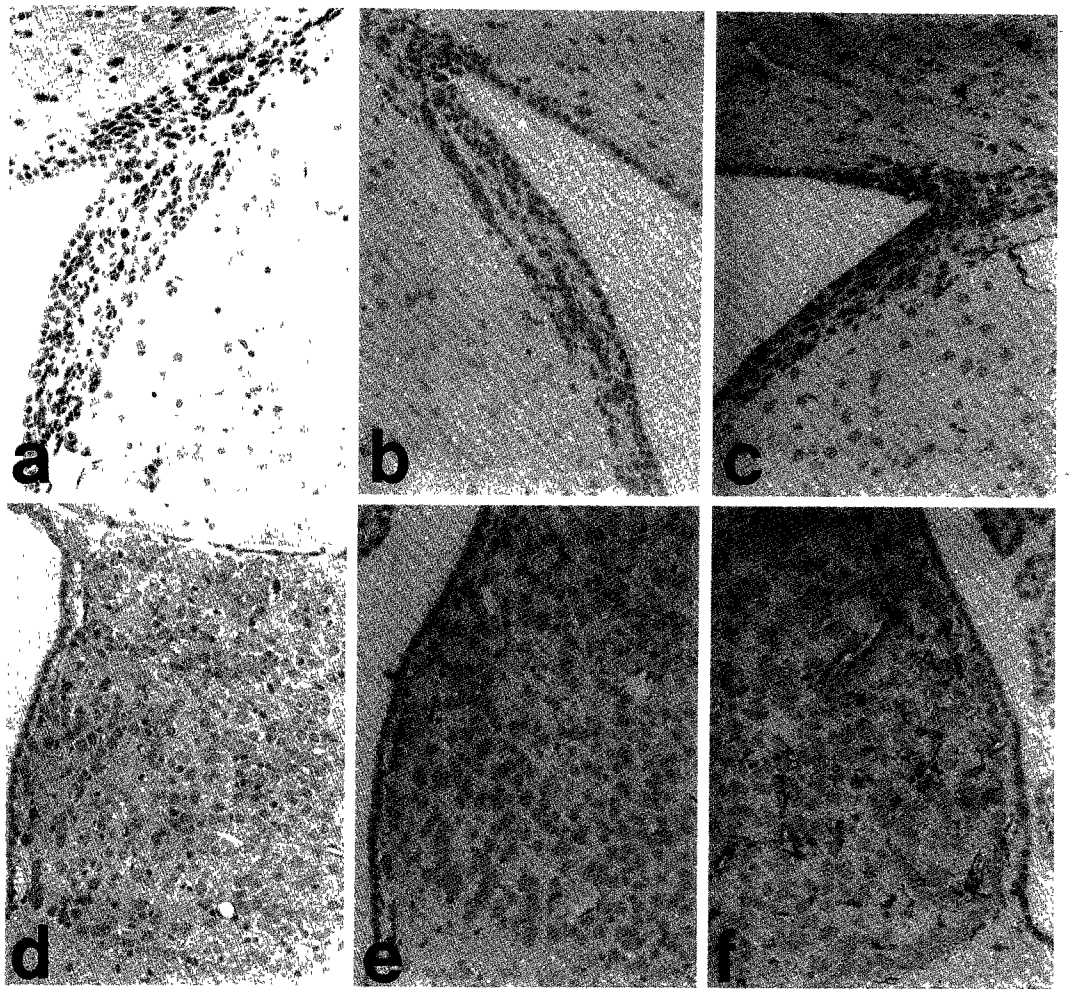

Fig. 3. Subependymal plate(a-c) and medial habenular nucleus(d-f) in normal control rat
a : Actively proliferating primitive cells in subependymal plate with mitosis(H-E, ×50)
b : Negative reaction of primitive cells with PKC γ antibody compared to positive reaction in ependymal lining cells(PAP, ×50)
c : Strong positive reaction of primitive cells and ependymal lining cells with GFAP antibody(PAP, ×50)
d : More differentiated cells in medial habenular nucleus(H-E, ×50)
e : Positive reaction with PKC γ antibody in background and ependymal lining cells(PAP, ×50)
f : Mild positive reaction with GFAP antibody in cells of habenular nucleus and negative reaction in ependymal lining cells(PAP, ×50)

increased(average 50gms) at 3 months compare to 120-150 gm increase in control rats. As expected, the white matter showed no changes up to 3 months in this study, which was similar to previous studies[1)19)-21)].

The epithelial cells of choroid plexus showed mild to moderate vacuolation(25~75% of rats) with mild to moderate perivascular edema(25% of rats) starting from 10 min up to 9 days and at 3 months, all 5 rats (100%) showed moderate vacuolation of epithelial cells as well as considerable vascular dilatation. Previous study done on rats killed during first post-irradiation month following various doses of 20 to 30Gy X rays showed marked vacuolation of epithelial cells with diffuse perivascular edema of neuropil at 4 days after irradiation[22)]. The changes observed in this study was much less than that study, which could be

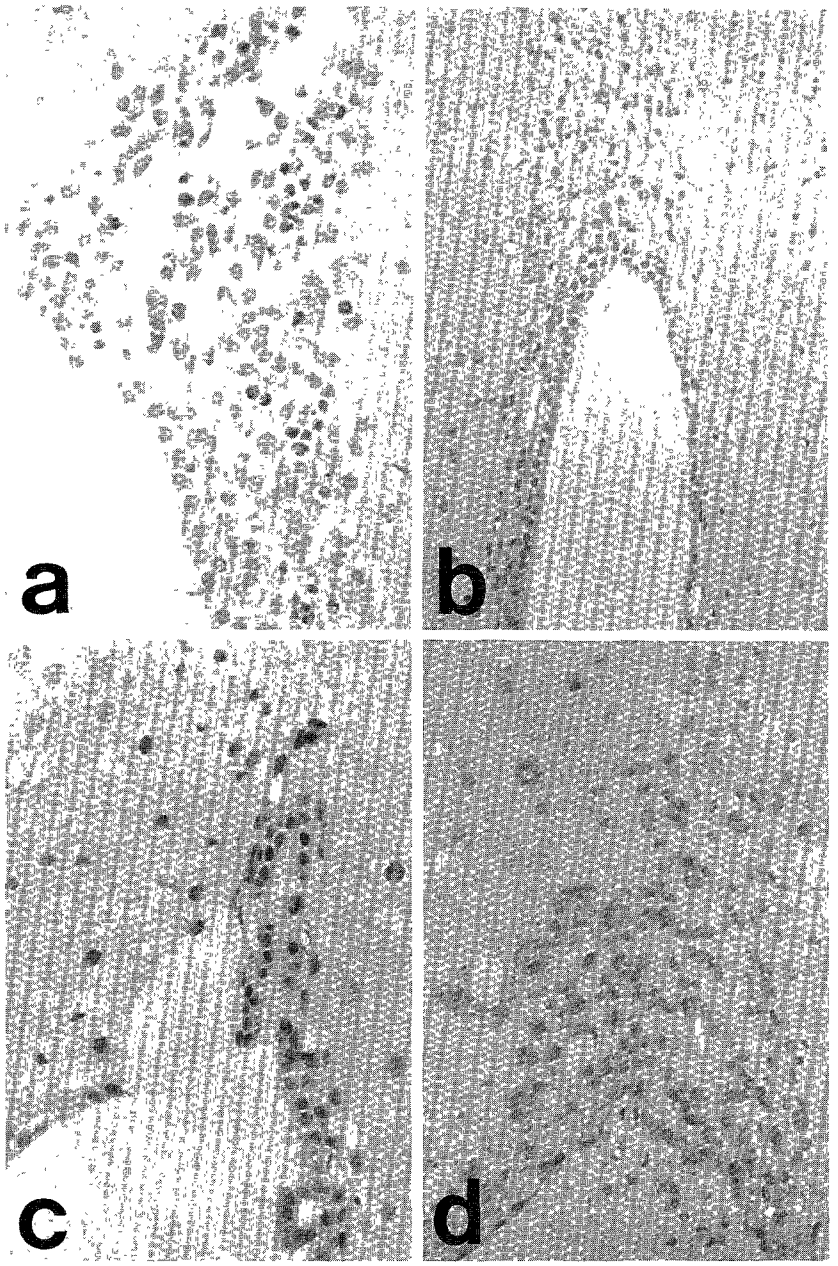

Fig. 4. a : Subependymal plate with numerous apoptosis at 3 hours postirradiation(H-E, ×100).
b : Markedly decreased number of cells in subependymal plate at 3 days postirradiation(H-E, ×50).
c : Absence of cells in subependymal plate and dilated ventricle at 3 months postirradiation(H-E, ×100).
d : Cellularity of subependymal plate in age-matched control(H-E, ×80).

explained by morbid status. The changes of PKC γ IR in epithelial cells were not discernible quantitatively according to the time in this study, but showed marginal displacement after the irradiation. This displacement of PKC could be either secondary change due to vacuolization of the cytoplasm or the ex-

Table 1. Post-irradiation changes of subependymal primitive cell layer

P-i time	Locat	Degen (% of rat)	Apoptosis (% of rat)	Cell Dens (%)	GFAP
control	spc	0	0	100	2+
	Mhb	0	0	100	1+
10 min	spc	1+(100)	1+(75)	100	2+
	Mhb	0	0	100	1+
1 hour	spc	1+(100)	1+(100)	100	2+
	Mhb	0	0	100	1+
3 hours	spc	3+(100)	3+(100)	100	-
	Mhb	1+(50)	0	100	-
1 day	spc	1+(100)	1+(100)	< 25	3+
	Mhb	1+(100)	0	100	2+
3 days	spc	1+(100)	1+(50)	< 25	3+
	Mhb	1+(100)	0	100	2+
9 days	spc	0	0	< 10	3+
	Mhb	1+(100)	1+(50)	100	1+
3 months	spc	0	0	0	3+
	Mhb	1+(100)	0	> 90	2+

P-i : post-irradiation Spc : subependymal primitive cell Mhb : medial hebenular nucleus
Locat : location Degen : degeneration Dens : density GFAP : glial fibrillary acidic protein

pression of enzyme activation[10)-16)] or both.

The blood vessels did not show considerable changes except mild to moderate perivascular edema during early stage(up to 9 days) in this study and were moderately dilated at 3 months with no evidence of fibrinoid necrosis of vascular wall or vascular thrombosis, which was similar to previous studies[23)-25)]. Reinhold and Hopewell[4)] reported gradual development of vascular abnormalities between 12 and 14 months after irradiation. A histological assessment of time- and dose-related changes in choroid plexus after local irradiation of rat brain with single dose of 17.5~25Gy showed atrophy of the epithelial layer after 13 weeks with recovery by 39 weeks after irradiation[26)]. Endothelial cell reduction was more prominent and progressive and effects of irradiation on the endothelium was considered more important than that to the epithelial cells. The results from this study could not be compared with previous studies, since the observation times were different.

Subependymal plate is a layer of mitotically active cells, from which cells continue to migrate out during adult life and differentiate into neuroglia[27)28)]. Local stem cell depletion and loss of functional subunits were also proposed as mechanism of irradiation encephalopathy[29)-31)]. The principal biologic changes of cell division by irradiation include delay of cell cycle, cell death during or after mitotic divisions, or nonlethal damage to chromosomes[32)]. In this study, the control brains showed several mitotic figures in SEP with no apoptosis. Apoptosis was first noted at 10 min post-irradiation and increased at 1 hour and maximum at 3 hours and decreased thereafter accompanied by decreased numbers of primitive cells. Cell density was markedly decreased to 25% of normal control at 1 and 3 days and less than 10% at 10 days, and totally absent at 3 months post-irradiation. The results were different from previous studies done on radiation effects on SEP. Hopewell and Cavanagh[2)] counted mitotic activity in subependymal plate cells about the lateral ventricles at various times after 2, 8, 20, and 40Gy irradiation and showed dose-dependent changes in mitotic activity. Mitotic counts declined rapidly in the first 24 hours after irradiation, followed by a rise to a peak at 7 days and further decline up to 14 days and gradual return by 3 months to within normal limit with the doses of 20Gy or less. The re-

covery to a normal mitotic count was also dose-dependent ; 1 month after 2Gy of local irradiation compared to 3 months after 20Gy. With 40Gy irradiation, no recovery to within normal limit was observed and 6 months after irradiation the mitotic count was zero. They did not recognize the apoptosis at that time and the difference in cell density at 3 months post-irradiation could be explained by difference in radiation method.

Apoptosis was introduced in 1972 and has been known as a distinctive and important mode of cell injury[33)-37)]. Apoptosis is thought to be responsible for numerous physiologic and pathologic events including programmed cell death during embryogenesis, hormone-dependent involution in the adult, cell deletion in proliferating cell populations, cell death in tumors, death of immune cells, pathologic atrophy of hormone-dependent tissues, and cell death produced by a varitey of injurious stimuli such as mild thermal injury, radiation, cytotoxic anticancer drugs, and possibly hypoxia[34)37)38)]. Apoptosis depends on gene activation and new protein synthesis[39)]. Apoptosis-specific genes that stimulate (*ced-3,4*) or inhibit (*ced-9*) cell death were identified in developing nematode *C. elegans*[40)]. Certain oncogenes and suppressor genes playing a regulatory role in the induction of apoptosis include *bcl-2 oncogene*, which inhibits apoptosis induced by hormones and cytokines and *c-myc oncogene*, whose protein product can stimulate either apoptosis or cell growth[41)42)]. In addition, *p53*, normally stimulates apoptosis, but when mutated or absent, favors cell survival. Following DNA damage by irradiation, *p 53* is required for apoptosis[43)44)] compared to *p53* independent apoptosis induced by glucocor-ticoids or aging. Apoptosis plays an important role in regulation of tumor growth and tumor response to various forms of cancer therapy including radiotherapy and chemotherapy. Apoptosis develop rapidly, within hours, after cytotoxic treatments and apoptotic response correlates well with the antitumor efficacy of radiation and chemotherapy.

In this study, apoptosis was most prominent in primitive cells in SEP, starting as early as 10 min post-irradiation and maximum at 3 hours and decreased thereafter. The cellularity was markedly decreased as a result of apoptosis. Apoptotic cells were also noted in DG of hippocampus at 3 hours after the irradiation and in CA2~3 at 3 days. The cells in Mhb were more differentiated compared to those in SEP and showed only several apoptotic cells at 10 days and the cellularity was well preserved at 3 months. These findings showed positive relationship between the differentiation of cells and cellularity and the level of apoptosis. Previous study in developing nervous system showed that the acute cell injury in embryonic telencephalon by doses as low as 0.1Gy was not reversed up to 6 hours and injured cells expressed apoptotic death, which began at 2 hours after exposure and peaked at 6~9 hours[45)]. They also showed radiation-induced cell death in external granular layer of cerebellum in newborn mice exposed to 0.24Gy was suppressed completely by cycloheximide, a protein synthesis inhibitor. Further study on relevant gene expression and apoptosis in radiation encephalopathy will be helpful to understand the pathophysiology.

In this study, SEP showed increased PKC γ IR after 3 hour to 3 month, which was consistent with previous studies showing induced PKC expression by irradiation[14)-17)]. In irradiation induced programmed cell death, PKC has been known to be involved in signal transduction from the cellular membrane to the nucleus, where DNA fragmentation is induced[46)]. Contrast to SEP, Mhb and other structures of the brain showed transient increase IR at 3 days and no discernible changes, respectively. The findings of PKC IR as well as apoptosis were consistent with the relationship between selective radiation sensitivity of cells in SEP and relative tolerance of other structures. Increased GFAP IR in SEP after 1 day was consistent with reactive astroglial proliferation after neuronal loss.

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