Animals

Four-week-old male closed-colony CD-1 (ICR) mice (n=32) and inbred mice, i.e., C57BL/6N (n=11), C3H/HeN (n=33), BALB/cAnN (n=22), DBA/2N (n=33) and CBA/JN (n=22) mice, were purchased from Charles River Japan (Yokohama, Japan). All animals were housed in facilities that conformed to national guidelines for animal care. Experiments were performed following the 1987 guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animal Science.

Histological evaluation

The *C. albicans* extract was injected intraperitoneally to the mice in a volume of 0.2 ml on each of five consecutive days in the 1st and 5th weeks. At week 8, the mice were killed with carbon dioxide asphyxiation. At that time, sections of the heart, lung, liver, spleen, kidney, the hind limb, spine and testis were obtained. Tissue specimens were fixed in 10% formalin and embedded in paraffin. Hematoxylin and eosin (H&E)- and elastica van Gieson (EvG)-stained sections were prepared using routine histological techniques for examination by light microscopy. For the coronary arteries, step sections in the horizontal direction were made every 20 µm. All slides were investigated histologically for the presence of arteritis. Arteritis was defined as cell infiltration in all layers of the arteries regardless of destruction of the internal and external elastic lamina. Individuals with cell accumulation in the intima or minimal perivascular cell infiltration were rated as negative for arteritis.

Statistical analysis

Fisher's exact probability test was used to analyze the differences in the incidence of arteritis among the inbred mouse strains. A value of $p<0.05$ was considered statistically significant.

Results

Histopathological findings in CD-1 mice

Arterial lesions: Arteritis had developed in 21 of 32 (66%) CD-1 mice after the intraperitoneal injection of the *C. albicans* extract. In particular, the coronary arteries and aortic root close to the orifice of coronary arteries were the most frequently involved, showing an incidence of 50% (16 of 32 mice). The coronary artery lesions were localized in the proximal region of the extramural arteries, and no cell infiltration was observed in the intramural coronary arteries. Arteritis was observed at non-coronary artery sites in 8 of 32 (25%) mice, 15 arterial lesions in total (kidney 8, retroperitoneum 4, abdominal aorta 1, hilus of the liver 1, and spine 1). Histologically, the typical panarteritic lesion showed proliferative and granulomatous inflammation accompanied by numerous macrophages, lymphocytes, plasma cells and neutrophils. Marked fibrocellular intimal thickening with destruction of the internal elastic lamina and media was also observed (Fig. 1, 2). However, fibrinoid necrosis was only

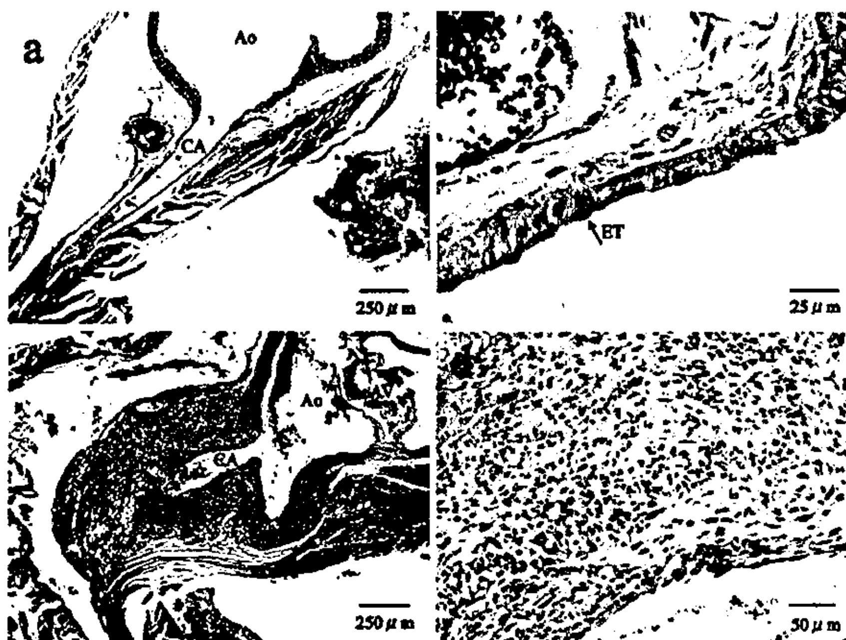

Fig. 1. (a) and (b): Histology of a normal coronary artery in CD-1 mouse. (a): Low power view, coronary artery branches off from the root of aorta. (b): High-power view, the artery wall mainly consists of smooth muscle cells (media). Endothelial cells line up at the luminal side of the artery. However, neither intimal thickening nor inflammatory cell infiltration is observed. (c) and (d): Histological features of the coronary arteritis induced in a CD-1 mouse by intraperitoneal injection of *C. albicans* extract. (c): Low power view, severe inflammation involves coronary artery and aortic root. Proximal site of the coronary artery is slightly dilated (☆). (d): High-power view; productive and granulomatous panarteritis accompanied by numerous large mononuclear cells, lymphocytes, plasma cells and neutrophils is observed at the coronary artery. (H&E) Ao: Aorta, AV: Aortic valve, CA: Coronary artery, ET: Endothelial cell

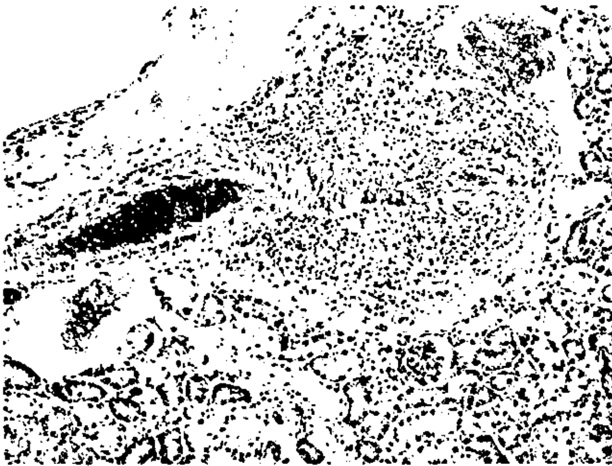

Fig. 2. Granulomatous inflammation in an artery in the kidney of a CD-1 mouse induced by intraperitoneal injection of *C. albicans* extract. (H&E)

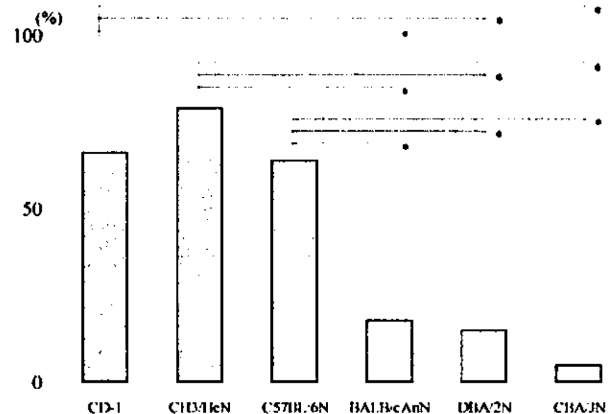

Fig. 4. Differences in incidence of arteritis among inbred mice. A closed-colony CD-1 mouse and five inbred mouse strains were classified into two groups according to the incidence of arteritis. Vasculitis-resistant strains; BALB/cAnN, DBA/2N and CBA/JN, Vasculitis-susceptible strains; CD-1, C3H/HeN and C57BL/6N mice. * $p < 0.01$

rarely noted in the arterial lesions. Meanwhile, in mild arterial lesions, small numbers of large mononuclear cells, lymphocytes and neutrophils were observed mainly in the media and adventitia, but neither intimal thickening nor destruction of the internal or external elastic lamina was noted.

Non-arterial lesions: Foamy macrophages appeared in the marginal sinus of the lymph nodes in the retroperitoneum and mediastinum. In addition, necrosis with karyorrhexis was seen in the subcortical area of lymph nodes (Fig. 3a, b). In the liver, scattered microabscesses accompanied by eosinophilic liver cell necrosis (Fig. 3c) and small granulomatous lesions were observed (Fig. 3d).

Differences in incidence of arteritis among inbred mice

The five inbred mouse strains other than CD-1 mouse that were tested fell into two sharply separated groups. For the first group of strains, classified as susceptible to vasculitis (C3H/HeN, C57BL/6N), the incidence of vasculitis was 79% (26 of 33 mice) and 64% (7 of 11 mice), respectively. In the second group of strains, classified as resistant to vasculitis (BALB/cAnN, DBA/2N and CBA/JN), the incidence of vasculitis was much lower: 18% (4 of 22 mice), 15% (5 of 33 mice) and 5% (1 of 22 mice), respectively (Fig. 4). The coronary arteries and aortic root were the most frequently

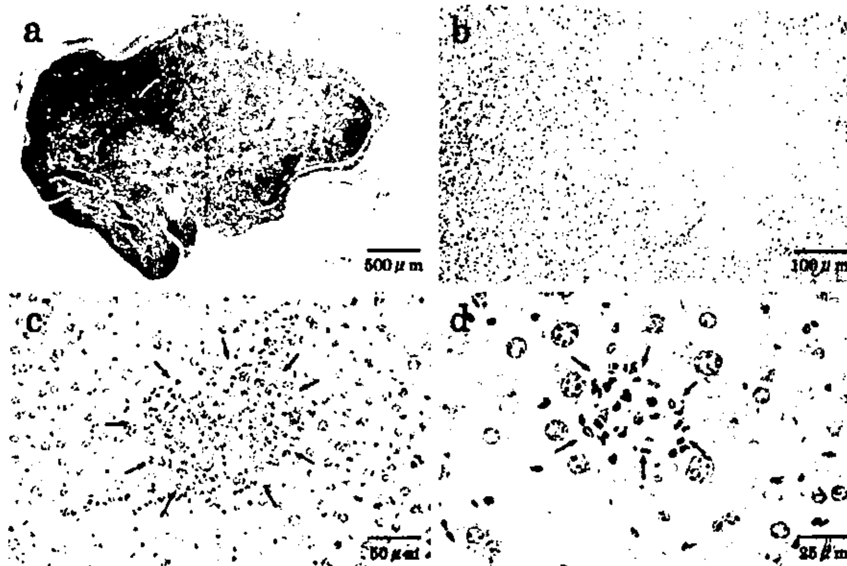

Fig. 3. Histological features of non-arterial lesions seen in a CD-1 mouse induced by intraperitoneal injection of *C. albicans* extract. (a) and (b): Lymph-node in the mediastinum. Large coagulative necrosis containing fragmented nuclei was located in the subcortical region of the lymph-node (arrow in (a)). (c) and (d): Liver. Microabscess with eosinophilic liver cell necrosis (arrow in (c)) and small granuloma composed of histiocytes and a small number of lymphocytes (arrow in (d)) lay scattered in the liver. (H&E)

Table 1. Incidence and distribution of arteritis in CD-1 mice and selected inbred mouse strains following injection of *C. albicans* extract.

Mouse strain	H-2 Haplotype[a]	n[b]	Arteritis[c]	Distribution				
				Heart[d]	Kidney	Retro-peritoneum	Abdominal aorta	Others
CD-1		32	21 (66%)	16 (50%)	8 (25%)	4 (13%)	1 (3%)	2 (6%)[e]
C3H/HeN	k	33	26 (79%)	24 (73%)	1 (3%)	4 (12%)	0	0
C57BL/6N	b	11	7 (64%)	7 (64%)	0	1 (9%)	0	0
BALB/cAnN	d	22	4 (18%)	4 (18%)	0	0	0	0
DBA/2N	d	33	5 (15%)	5 (15%)	0	0	1 (3%)	1 (3%)[f]
CBA/JN	k	22	1 (5%)	0	0	0	1 (5%)	0

[a] Histocompatibility-2 haplotype, [b] Number of mouse examined, [c] Number of mouse with arteritis, [d] Coronary artery and/or aortic root, [e] Artery in the hepatoportal and peritesticular region, [f] artery in the testis.

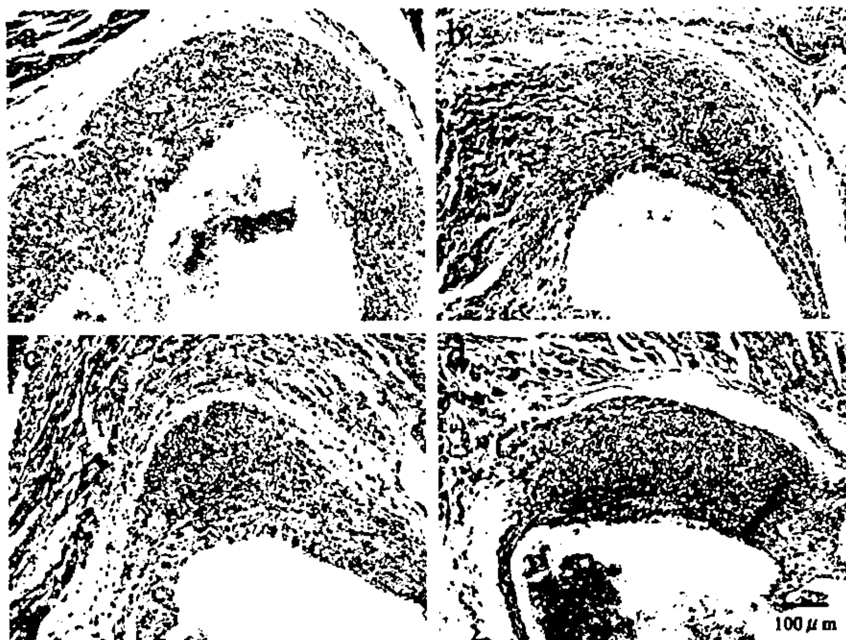

Fig. 5. Comparison of histology of the inflammation at the aortic root. Inflammatory cells infiltrate the aortic wall, but no striking histological differences were noted between the inbred mouse strains. (a) C3H/HeN, (b) C57BL/6N, (c) BALB/cAnN, (d) DBA/2N. (H&E).

involved, while the arteries in the kidney, retroperitoneum, aorta and testis were rarely involved (Table 1). The histopathological findings of the arteritis were very similar among the five inbred mouse strains (Fig. 5). No obvious differences were noted in the light microscopic examination.

Discussion

We described the histopathological changes that occurred after intraperitoneal injection of a *C. albicans* extract to mice. Systemic vasculitis developed in 66% of the CD-1 mice. Of particular note was that the arteritis most frequently involved the coronary arteries, while other arteries such as the renal arteries, small arteries in the retroperitoneum and peritesticular region were also involved. The histological features were very distinctive; that is, the arterial lesions showed proliferative and granulomatous inflammation without fibrinoid necrosis. To date, animal vasculitis models such as in MRL/lpr mice and (NZBxNZW) F1 mice, which spontaneously develop systemic vasculitis resembling that in collagen disease, have been actively analyzed [5–7]. On the other hand, coronary arteritis-induced animal models are created by treatment with a foreign serum, *Lactobacillus casei* cell wall extract, etc., to induce vasculitis [8–10]. However, histologically, these vasculitis models are characterized by necrotizing vasculitis with fibrinoid degeneration, which is conclusively different from our vasculitis model. Thus, it could be said that the vasculitis mouse model described here is a very unique, ingenious experimental model. The histological features of the vascular lesions in this model are similar to those in human Kawasaki disease.

The biggest problem of Kawasaki disease is that coronary artery aneurysms often develop following the coronary arteritis. Thrombotic occlusion of the coronary aneurysms leads to ischemic heart disease in children. The characteristic pathological feature of Kawasaki disease arteritis in the acute phase is granulomatous inflammation composed mainly of large mononuclear cells [11, 12]. The structure of the arteries, such as the media, internal and external elastic lamina, is completely destroyed at the aneurysms, but fibrinoid necrosis is seldom seen. We note that in our model the coronary artery is also dilated, and that the most important factor causing this was destruction of the media and internal elastic lamina due to inflammatory cell infiltration [13]. The distribution and incidence of arterial lesions in Kawasaki disease autopsy patients were reported as follows: coronary arteries, 95%; kidney, 75%; lung, 45%; and ovary or testis, 20–30% [11]. Thus, even in these points, our model is similar to Kawasaki disease. We think that, from the histological viewpoint, our animal model can be considered to be a model of Kawasaki disease.

Another objective of the present study was to investigate the participation of genetic factors in the development of vasculitis by using different inbred mouse strains as the first step to examine the susceptibility loci. Five inbred mouse strains, after intraperitoneal injection of *C. albicans* extract, were classified as either resistant (CBA/JN, DBA/2N and BALB/cAnN) or susceptible (C3H/HeN and C57BL/6N). DBA/2N and BALB/cAnN mice, whose histocompatibility-2 (H-2) haplotype were H-2*d* [14], were resistant to vasculitis. However, the susceptibility to vasculitis between CBA/JN and C3H/HeN mouse was apparently different in spite of the same H-2*k* haplotype [14]. These findings suggest that genetic control of the susceptibility of mice to vasculitis induced by the *C. albicans* extract is not linked to the H-2 loci. Nose et al. indicated that systemic vasculitis in MRL/lpr mice is genetically controlled by the cumulative effects of multiple gene loci [15, 16]. We tried to clarify the coronary arteritis susceptibility loci by backcrossing high-incidence and low-incidence mouse strains. To date, several possible candidate loci which regulate some inflammatory cytokines have been found (papers in preparation).

From a different point of view, recent work has demonstrated that the Th1-Th2 balance appears to be a key factor in the healing or progression of some infectious diseases [17]. Interestingly, it has also been found that BALB/c mice, which have Th2-dominant immunity, are susceptible to *Leishmania major* infection, while C57BL/6 mice and C3H/HeN, which have Th1-dominant immunity, are resistant to *L. major* infection [18]. In addition to BALB/c mice, CBA/J mice showed a significant elevation of serum total IgE after nasal challenge with *Schistosoma mansoni* egg antigen, but no such increase in serum total IgE was seen in C57BL/6 mice [19]. Conversely, Tanaka et al. [20, 21] reported that Th1-dependent mouse liver injury was induced by combination treatment with *Propionibacterium acnes* and lipopolysaccharide. They found that BALB/c and DBA/2 mice were resistant to liver injury, while C57BL/6 mice were susceptible. We demonstrated that C57BL/6N and C3H/HeN mice are susceptible to *C. albicans* extract-induced vascular injury, whereas BALB/cAnN, DBA/2N and CBA/JN mice are resistant. This suggests that in our mouse model the Th1-dominant immune response to injection of *C. albicans* extract is closely related to the vascular injury. Some reports indicate that cytokine overproduction might explain the development of a Th1 response to *C. albicans* infection [22, 23]. It has also been suggested that a Th1-dominant immune response is induced by *C. albicans* cell wall mannoprotein [24]. Mannoprotein is the principal component of the preparation used in the present study, and the extract also contains a small amount of β1,3- and β1,6-glucan. Our results about analysis of the structure and biological activity of the *C. albicans* extract suggested mannan and β1,6-glucan were strongly involved in the development of the arteritis [25]. These components may induce a Th-1 dominant immune response even in this tissue injury model. Serial sacrifice studies in our recent experiments indicated that the serum levels of IFN-γ, IL-12 and TNF-α increased immediately after injection of the *C. albicans* extract. Furthermore, our immunohistochemical study using frozen sections found that cells positive for antibodies against IFN-γ and IL-12, in addition to CD11b, mouse neutrophils, CD68, CD3 and CD20, were present in the vasculitic lesions (papers in preparation).

Here, we presented the histological details on the only vasculitis murine model which closely resembled Kawasaki disease in humans and the differences in arteritis susceptibility among inbred mouse strains, which will serve as a foundation for discussing the results of further, ongoing examinations. Carefully designed studies using this animal model may provide fundamental information which will increase our understanding of the pathogenesis, natural history and appropriate therapy for coronary arteritis in humans.

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p53 Deficiency Rescues Neuronal Apoptosis but Not Differentiation in DNA Polymerase β-Deficient Mice

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In mammalian cells, DNA polymerase β (Polβ) functions in base excision repair. We have previously shown that Polβ-deficient mice exhibit extensive neuronal cell death (apoptosis) in the developing nervous system and that the mice die immediately after birth. Here, we studied potential roles in the phenotype for p53, which has been implicated in DNA damage sensing, cell cycle arrest, and apoptosis. We generated Polβ$^{-/-}$ p53$^{-/-}$ double-mutant mice and found that p53 deficiency dramatically rescued neuronal apoptosis associated with Polβ deficiency, indicating that p53 mediates the apoptotic process in the nervous system. Importantly, proliferation and early differentiation of neuronal progenitors in Polβ$^{-/-}$ p53$^{-/-}$ mice appeared normal, but their brains obviously displayed cytoarchitectural abnormalities; moreover, the mice, like Polβ$^{-/-}$ p53$^{+/+}$ mice, failed to survive after birth. Thus, we strongly suggest a crucial role for Polβ in the differentiation of specific neuronal cell types.

Repair of DNA damage is essential for maintaining the integrity of the genetic information necessary for normal development and physiological consequences (28). DNA polymerase β (Polβ) is a 39-kDa protein of a single polypeptide, consisting of two catalytic, functional domains. The N-terminal 8-kDa domain carries a 5′-deoxyribose phosphate lyase activity, whereas the C-terminal 31-kDa domain carries a polymerase activity that fills a short gap with a 5′-phosphate (26, 40). Polβ is a critical component of the base excision repair (BER) pathway. The BER pathway repairs DNA damage, such as apurinic/apyrimidinic (AP) sites and base modifications, which spontaneously occur or are induced by a variety of endogenous and exogenous agents, including reactive oxygen species and DNA alkylating agents. Biochemical studies have identified two types of BER in mammalian cells: a short-patch pathway involving replacement of one nucleotide and a long-patch pathway involving gap-filling of several nucleotides (46). The BER pathway is generally initiated by a specific DNA glycosylase that recognizes and removes a damaged base to generate an AP site in DNA, followed by incision of the site by an AP endonuclease. In the short-patch BER pathway, Polβ removes the 5′-deoxyribose phosphate and fills the single nucleotide gap, and finally DNA ligase I or a complex of XRCC1 and DNA ligase III ligates the nick. On the other hand, the long-patch BER pathway requires proliferating cell nuclear antigen (PCNA), flap endonuclease 1 (FEN-1), and DNA ligase I to excise a flap-like structure resulting from strand displacement by Polβ and/or Polδ/ε and to ligate the nick (6).

We and others previously showed that Polβ-deficient mice exhibit a reduced size and weight and die with a respiratory defect immediately after birth (13, 42). In Polβ-deficient mice, extensive cell death (apoptosis) occurs in postmitotic neurons in the developing central and peripheral nervous systems (42). This neuronal apoptosis is closely associated with the period between the onset and cessation of neurogenesis. Abnormalities in embryonic tissues other than the nervous system have not yet been reported (13, 19, 21). Therefore, we suggested that Polβ plays an essential role specifically in the development of the nervous system (42). However, the cause of this neuronal apoptosis remains entirely unknown. Mouse embryonic fibroblast cells in culture, derived from a Polβ knockout mouse, are viable and show normal growth characteristics (41). Although the mutant cells exhibit BER defects, as evidenced by increased sensitivity to DNA alkylating agents, their cell extracts still retain an activity to repair a damaged base residue in DNA substrate (6), indicating that there are both Polβ-dependent and -independent BER pathways in vivo.

The tumor suppressor protein p53 plays a prominent role in the maintenance of genomic integrity (27). It is activated by different types of DNA damage, including single-strand breaks (SSBs), double-strand breaks (DSBs), and adducts, which are generated by endogenous or exogenous mechanisms. The activated p53 has a choice of cell cycle arrest for repair or apoptosis, depending on the level of damaged DNA; i.e., unless the damage is repaired, p53 leads to apoptosis. Recent studies show that p53 directly interacts with Polβ, stimulating BER activity (37, 50). In the nervous system, it has been shown that p53 regulates neuronal apoptosis after neuronal injury induced by excitotoxins, hypoxia, and ischemia that cause oxidative damage (3, 29, 33, 47). In p53-deficient mice, kainic acid exci-

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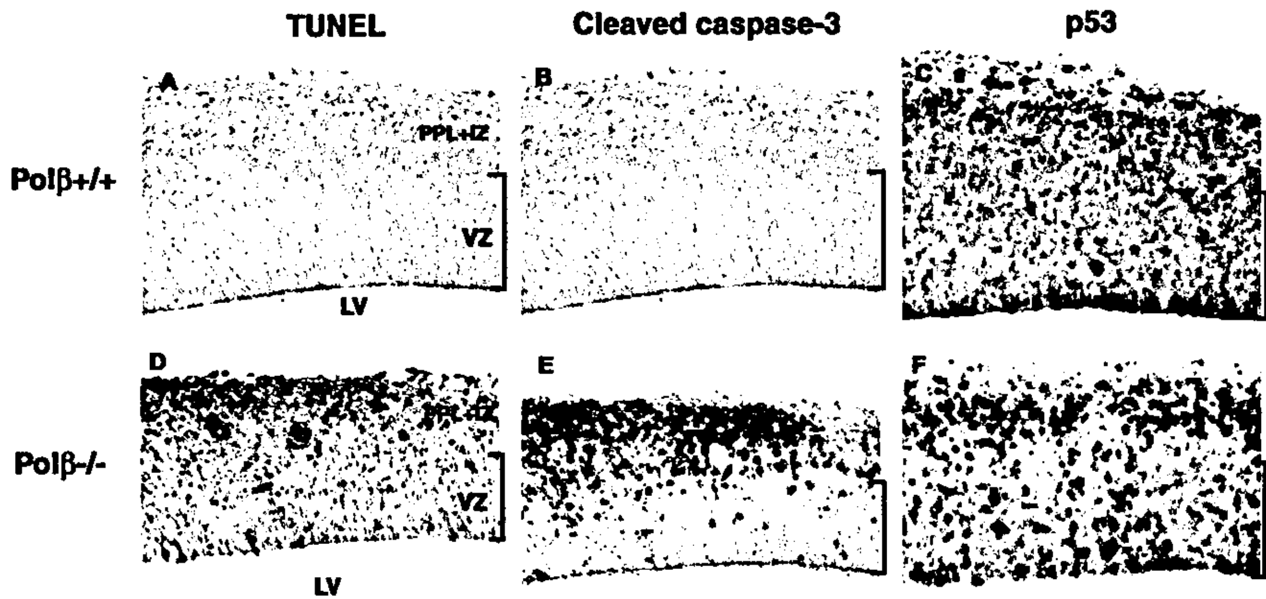

FIG. 1. Polβ deficiency induces apoptosis and activates p53 in the developing nervous system. Coronal sections of E13.5 developing neocortices in Polβ$^{+/+}$ (wild-type) (A to C) and Polβ$^{-/-}$ (D to F) embryos were assayed by TUNEL staining (A and D) or immunohistochemistry with anti-cleaved caspase-3 antibody (B and E) and anti-p53 antibody (C and F). LV, lateral ventricle.

totoxicity- or ischemia-induced brain damage is significantly reduced (10, 34). These observations suggest the involvement of p53 in the control of neuronal apoptosis.

A close link between DNA damage and neurodegeneration appears evident from many pathological data and observations with mouse knockouts (8, 38). In mice deficient in DNA ligase IV (Lig4) and XRCC4, the main components of nonhomologous-end-joining apparatus for DSB repair, differentiating neurons undergo massive cell death (4, 14, 17, 22). However, this apoptosis is completely rescued by p53 deficiency (15, 16). It would be important to examine whether neuronal cell death found in Polβ-deficient mice (42) is mediated by p53 activation. Here we study a potential role for p53 in the phenotypes, including neuronal cell death associated with Polβ deficiency. We found that p53 deficiency rescues the neuronal apoptosis in a Polβ-deficient background. However, it should be noticeable that Polβ$^{-/-}$ p53$^{-/-}$ mice still exhibit cytoarchitectural defects in the development of the nervous system and die shortly after birth. These observations strongly suggest that Polβ is crucial for the differentiation process of specific neuronal cell types.

MATERIALS AND METHODS

Mice. Polβ-deficient mice were described previously (42). p53-deficient mice (C57BL/6J-Trp53 tm1Tyj) were obtained from The Jackson Laboratory (24). PCR genotyping protocol for p53 targeted allele are directed on the website of JAX MICE, The Jackson Laboratory (http://jaxmice.jax.org). Noon of the day on which the vaginal plug was detected in the morning was designated embryonic day 0.5 (E0.5). All mice were maintained in a pathogen-free environment under the guidelines of Kihara Institute for Biological Research, Yokohama City University, for laboratory animals.

Histology, immunohistochemistry, and TUNEL assay. Embryos were perfused with 4% paraformaldehyde and 7% picric acid in 0.1 M sodium phosphate buffer (pH 7.4); the brain was removed and postfixed in the same fixative for 2 h, equilibrated with 25% sucrose-phosphate-buffered saline, frozen in OCT compound (Sakura Finetechnical Co.) and sectioned on a cryostat (10 μm). The sections were incubated with rabbit anti-p53 polyclonal antibody CM-5 (Novocastra Laboratories, 1:3,000), rabbit anti-cleaved caspase-3 polyclonal antibody (Cell Signaling Technology; 1:100), mouse anti-PCNA monoclonal antibody PC10 (Sigma; 1:100), mouse anti-neuron specific type-III β-tubulin monoclonal antibody Tuj1 (BabCO; 1:1,000), mouse anti-phosphorylated neurofilament SMI31 (Sternberger Monoclonal Antibodies; 1:4,000), and rabbit anti-calbindin/spot 35 polyclonal antibody (a kind gift of T. Yamakuni) (1). Cy3 or horseradish peroxidase-conjugated antibody was used for a secondary antibody to visualize primary antibody. TSA Biotin System (Perkin-Elmer Life Sciences) was applied to anti-p53, anti-calbindin immunohistochemistry. The TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay was performed on cryosections by using 0.12 U of TdTase (Roche)/μl with 0.5 μM biotin-14-dATP (Invitrogen) in 1× TdT buffer (Roche) with 1.5 mM $CoCl_2$. Horseradish peroxidase-conjugated biotin (Jackson Immunoresearch Laboratories) was used for signal detection. Cell nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) for immunofluorescence. Cresyl violet (Sigma) staining was performed to show neuronal architecture.

Western blot analysis. Cell extracts were prepared from developing telencephalons in E13.5 embryos, electrophoresed in an 8.0% sodium dodecyl sulfate-polyacrylamide gel, and transferred to an Immobilon membrane (Millipore) as described previously (42). The membrane was probed with anti-human phospho-p53 (Ser-15) antibody (Cell Signaling Technology; 1:1,000) and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Chemicon) and detected with enhanced chemiluminescent detection reagents (ECL Plus kit; Amersham).

RESULTS

Polβ deficiency activates the p53-dependent apoptosis pathway. p53 is activated and stabilized after excessive DNA damage generated by endogenous or exogenous mechanisms, and induces apoptosis (27). We examined whether neuronal cell death found in Polβ-deficient mice (42) was induced by such p53 activation. To detect DNA fragmentation in cells undergoing apoptosis, we performed terminal deoxynucleotidyltransferase-mediated dUTP biotin nick-end labeling (TUNEL) assay. In Polβ$^{+/+}$ (wild-type) mice, TUNEL-positive cells were not detected in developing neocortex at E13.5 (Fig. 1A). In contrast, in Polβ$^{-/-}$ developing neocortex, an extensive number of TUNEL-positive cells were observed in

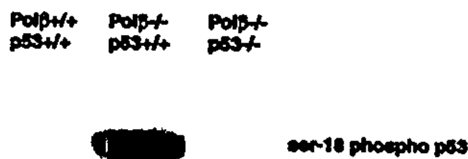

FIG. 2. Western blot analysis of phosphorylated serine-18 of p53 with cell extracts prepared from E13.5 developing telencephalons of Pol$\beta^{+/+}$ p$53^{+/+}$ (wild-type), Pol$\beta^{-/-}$ p$53^{+/+}$, and Pol$\beta^{-/-}$ p$53^{-/-}$ embryos. The same amounts of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-human phospho-p53 (Ser-15) antibody.

TABLE 1. Genotypic analysis of Pol$\beta^{+/-}$ p$53^{+/-}$ intercrosses

Age	No. of mice with genotype:									No. of litters	Total no.
	Pol$\beta^{+/+}$ p53			Pol$\beta^{+/-}$ p53			Pol$\beta^{-/-}$ p53				
	+/+	+/−	−/−	+/+	+/−	−/−	+/+	+/−	−/−		
E11.5	5	3	7	10	16	7	3	9	1	8	61
E13.5	6	11	2	12	18	10	4	10	4	9	77
E18.5	17	21	11	16	25	14	3	20	6	19	133
Weaned (4 wk)	13	16	8	9	14	6	0	0	0	14	66

the primordial plexiform layer (PPL) and intermediate zone (IZ), where postmitotic neuronal cells were present; but these cells were less detectable in the ventricular zone (VZ), where proliferating neuronal progenitor cells were present (Fig. 1D).

We next examined the expression of cleaved caspase-3 and p53 by immunohistochemistry in E13.5 developing neocortex. Since caspase-3 is activated by proteolytic cleavage of its inactive zymogen by apoptosis (35), cleaved caspase-3 is a useful marker for apoptotic cells. Anti-cleaved caspase-3 antibody could obviously detect apoptotic neuronal cells in Pol$\beta^{-/-}$ embryos (Fig. 1E), as observed by the above TUNEL assay, whereas wild-type embryos were not stained (Fig. 1B). Importantly, a larger number of cells with higher p53 levels were seen in Pol$\beta^{-/-}$ embryos than in wild-type (Fig. 1C versus 1F); in the VZ, such p53-stained cells were more frequent than the TUNEL- or cleaved caspase-3-positive cells. It is known that serine-15 of human p53 (equivalent to serine-18 in mouse p53) is phosphorylated in response to DNA damage (39). Therefore, we performed Western blot analysis with anti-phosphorylated serine-15 of human p53 specific antibody with cell extracts from E13.5 developing telencephalons of Pol$\beta^{+/+}$ p$53^{+/+}$ (wild-type), Pol$\beta^{-/-}$ p$53^{+/+}$, and Pol$\beta^{-/-}$ p$53^{-/-}$ embryos (generation of the double-mutant mice will be cited below). We found that the serine-18 of p53 in Pol$\beta^{-/-}$ p$53^{+/+}$ extracts was strongly phosphorylated compared to that in wild-type control, with no staining in Pol$\beta^{-/-}$ p$53^{-/-}$ extracts (Fig. 2). These results suggest an intriguing possibility that neuronal apoptosis observed in Polβ-deficient embryos is induced by p53 activation in response to DNA damage, immediately after final mitosis of the progenitor cells.

To explore potential physiological interactions between Polβ and p53, we produced mice null for both Polβ and p53. We bred Pol$\beta^{+/-}$ and p$53^{+/-}$ mice and generated Pol$\beta^{+/-}$ p$53^{+/-}$ double-heterozygous mice, which exhibited normal development and fertility. When Pol$\beta^{+/-}$ p$53^{+/-}$ mice were intercrossed, developing embryos with nearly the expected Mendelian ratios of all genotypes were observed at E11.5 to E18.5 (Table 1). However, no offspring with Pol$\beta^{-/-}$ p$53^{+/+}$, Pol$\beta^{-/-}$ p$53^{+/-}$, and Pol$\beta^{-/-}$ p$53^{-/-}$ genotypes was detected at weaning (ca. 4 weeks after birth). It should be noted that, like Pol$\beta^{-/-}$ p$53^{+/+}$ neonates, all Pol$\beta^{-/-}$ p$53^{+/-}$ and Pol$\beta^{-/-}$ p$53^{-/-}$ neonates died at postnatal day 1 (data not shown). With anti-cleaved caspase-3 antibody, we examined neuronal apoptotic phenotypes by staining sections of the developing telencephalon in E13.5 embryos and the spinal cord and dorsal root ganglion in E11.5 embryos of wild-type controls and littermates. In tissues of Pol$\beta^{-/-}$ $53^{+/+}$ mice, a large number of cleaved caspase-3-positive neuronal cells were observed compared to those of control mice (compare Fig. 3A and E with 3B and F). However, these stained cells were dramatically decreased in Pol$\beta^{-/-}$ p$53^{+/-}$ mice (Fig. 3C and G) and, more importantly, completely disappeared in Pol$\beta^{-/-}$ p$53^{-/-}$ double-mutant mice (Fig. 3D and H). These observations indicate that p53 deficiency rescues the neuronal apoptosis associated with Polβ deficiency and that p53 haploinsufficiency also substantially does so. These results indicate that the neuronal apoptosis in Polβ-deficient mice is mediated by the p53-dependent apoptosis pathway.

Proliferation and early differentiation of progenitors during neurogenesis appear normal in E13.5 Pol$\beta^{-/-}$ p$53^{-/-}$ mice. Proliferation and differentiation of progenitors are temporally and spatially controlled during neurogenesis (12). The generation of neurons from the progenitors involves successive steps in commitment and differentiation, which are progressively generating more restricted cell types. These steps include (i) cell type specification, (ii) exit from the cell cycle, (iii) differentiation into distinct cell types, (iv) migration into a correct destination, and (v) production of correct cell-cell contacts through dendritic and axonal processes. We examined effects of p53 deficiency on histogenesis of the neocortex by cresyl violet staining (Fig. 4A to F and 4P to R). In E13.5 wild-type lateral regions of the neocortex, generation of the PPL, IZ, and cortical plate (CP) was clearly observed (Fig. 4A), but the CP in the dorsal region was not (Fig. 4D). The ventrolateral-to-dorsomedial, morphological gradients are evident in the development of the neocortex (5). In contrast, in Pol$\beta^{-/-}$ p$53^{+/+}$ neocortices, the CP was not clearly seen due to an extraordinary number of both pyknotic and cleaved caspase 3-positive cells undergoing apoptosis (Fig. 4B, E, and H). However, in Pol$\beta^{-/-}$ p$53^{-/-}$ neocortices, the CP was almost normally generated (Fig. 4C), parallel with a striking disappearance of apoptotic cells (Fig. 4F and I). With the use of antibody against either PCNA, a proliferating cell marker (Fig. 4J to L), or neuron-specific type III β-tubulin, an early neuron marker (Fig. 4M to O), we observed the state of neuronal cells escaping from apoptosis in E13.5 developing neocortex. In the PPL and IZ of Pol$\beta^{-/-}$ p$53^{+/+}$ mice, expression of PCNA appeared in some apoptotic cells (Fig. 4K), whereas expression of type III β-tubulin was reduced with increasing apoptotic cells (compare Fig. 4N and M). Expression of PCNA is regulated by p53 in response to ionizing radiation in neuronal cells (45). Therefore, the PCNA activation in the apoptotic cells of Pol$\beta^{-/-}$

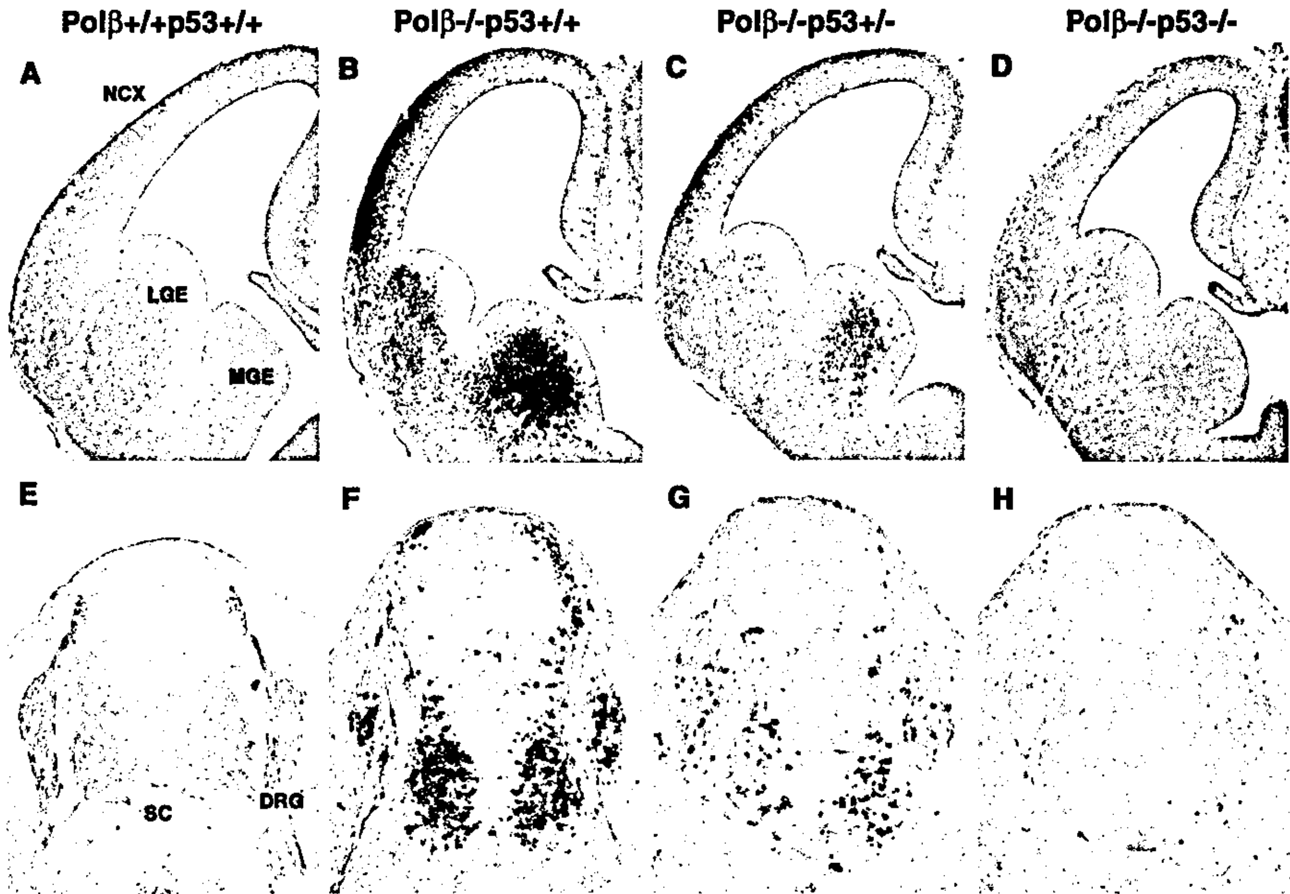

FIG. 3. Neuronal apoptosis in Polβ-deficient mice is mediated by the p53-dependent pathway. Coronal sections of E13.5 telencephalons (A to D) and E11.5 spinal cords and dorsal root ganglions (E to H) in Polβ$^{+/+}$ p53$^{+/+}$ (wild-type) (A and E), Polβ$^{-/-}$ p53$^{+/+}$ (B and F), Polβ$^{-/-}$ p53$^{+/-}$ (C and G), and Polβ$^{-/-}$ p53$^{-/-}$ (D and H) embryos were stained with anti-cleaved caspase-3 antibody. DRG, dorsal root ganglion; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; NCX, neocortex; SC, spinal cord.

p53$^{+/+}$ mice (Fig. 4K) might be due to a response to certain DNA damage. On the other hand, in Polβ$^{-/-}$ p53$^{-/-}$ mice, we found intact expression of both PCNA and type III β-tubulin, similar to wild-type controls (Fig. 4L and O, respectively). Neuronal progenitors expressing PCNA were restricted in the VZ (Fig. 4L); the differentiating neurons present in the PPL and IZ expressed type III β-tubulin at a normal level (Fig. 4O). Finally, at E14.5, formation of the CP in Polβ$^{-/-}$ p53$^{-/-}$ mice was more clearly observed than that in Polβ$^{-/-}$ p53$^{+/+}$ mice (compare Fig. 4R and Q). Together, it appears that in Polβ$^{-/-}$ p53$^{-/-}$ embryos early differentiation of neuronal cells escaping from apoptosis proceeds normally.

Formation of the nervous system is incomplete in E18.5 Polβ$^{-/-}$ p53$^{-/-}$ mice. p53 deficiency dramatically rescued neuronal apoptosis in Polβ-deficient mice (Fig. 3). As mentioned above, early neuronal differentiation appeared to proceed normally in E13.5 Polβ$^{-/-}$ p53$^{-/-}$ embryos (Fig. 4). As shown in Table 1, Polβ$^{-/-}$ p53$^{-/-}$ embryos could survive during gestation and died at postnatal day 1. Therefore, we examined the development of their brains at E18.5 by immunohistochemical analysis (Fig. 5). We observed slight but significant, neuronal defects in the telencephalon of Polβ$^{-/-}$ p53$^{-/-}$ embryos. Cresyl violet staining revealed that, compared to Polβ$^{-/-}$ p53$^{+/+}$ mice, the size of the telencephalon and its cytoarchitecture were moderately recovered in Polβ$^{-/-}$ p53$^{-/-}$ mice (compare Fig. 5B and C). We analyzed the axonal tract formation by staining phosphorylated neurofilaments. In Polβ$^{-/-}$ p53$^{+/+}$ brains, the major axonal tract, anterior commissure did not cross at the midline (Fig. 5E); notably, this defect could not be rescued by p53 deficiency (Fig. 5F). We also observed more aberrant axonal tracts in the striatum of Polβ$^{-/-}$ p53$^{+/+}$ brains (Fig. 5H) than in that of wild-type controls (Fig. 5G), and similar aberrations were observed in Polβ$^{-/-}$ p53$^{-/-}$ brains (Fig. 5I). These results indicate that, at least in some areas, the brain development in Polβ$^{-/-}$ p53$^{-/-}$ embryos was not complete. Recently identified is a cell type migrating from the lateral ganglionic eminence and the medial ganglionic eminence of the basal ganglia to the neocortex of telencephalons (2, 32). The tangentially migrating cells become interneurons synthesizing inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the CP of the neocortex. A great number of neuronal apoptotic cells were observed in both the lateral ganglionic eminence and the medial ganglionic eminence in Polβ$^{-/-}$ p53$^{+/+}$ embryos at E13.5 (compare Fig. 3A and B). We therefore examined whether GABAergic interneurons existed in the E18.5 neocortex. The interneurons in de-

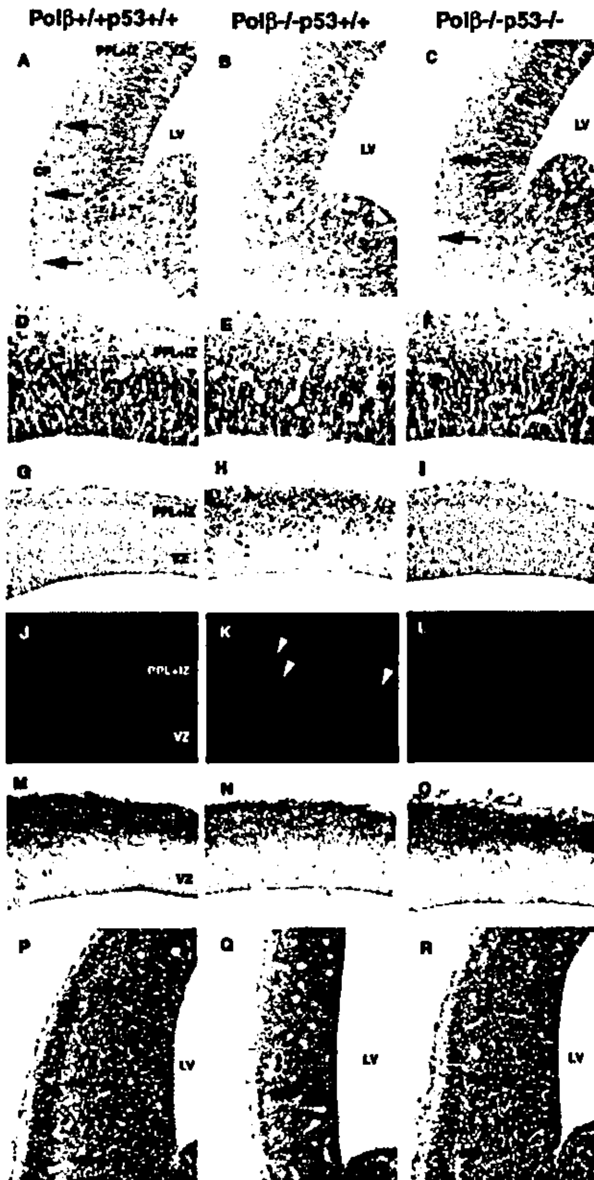

FIG. 4. Neuronal progenitors in E13.5 Polβ$^{-/-}$ p53$^{-/-}$ embryos appear to normally proliferate and differentiate. Coronal sections of telencephalons in Polβ$^{+/+}$ p53$^{+/+}$ (wild-type) (A, D, G, J, M, and P), Polβ$^{-/-}$p53$^{+/+}$ (B, E, H, K, N, and Q), and Polβ$^{-/-}$ p53$^{-/-}$ (C, F, I, L, O, and R) embryos at E13.5 (A to O) and E14.5 (P to R) were stained with cresyl violet (A to F and P to R). The sections were also stained with anti-cleaved caspase-3 antibody (G to I), anti-PCNA antibody (red) and DAPI (blue) (J to L), or anti-neuron specific type III β-tubulin antibody TuJ1 (M to O). Arrows in panels A, C, P, Q, and R indicate the CP. Arrowheads in panel K indicate PCNA-activated cells. LV, lateral ventricle.

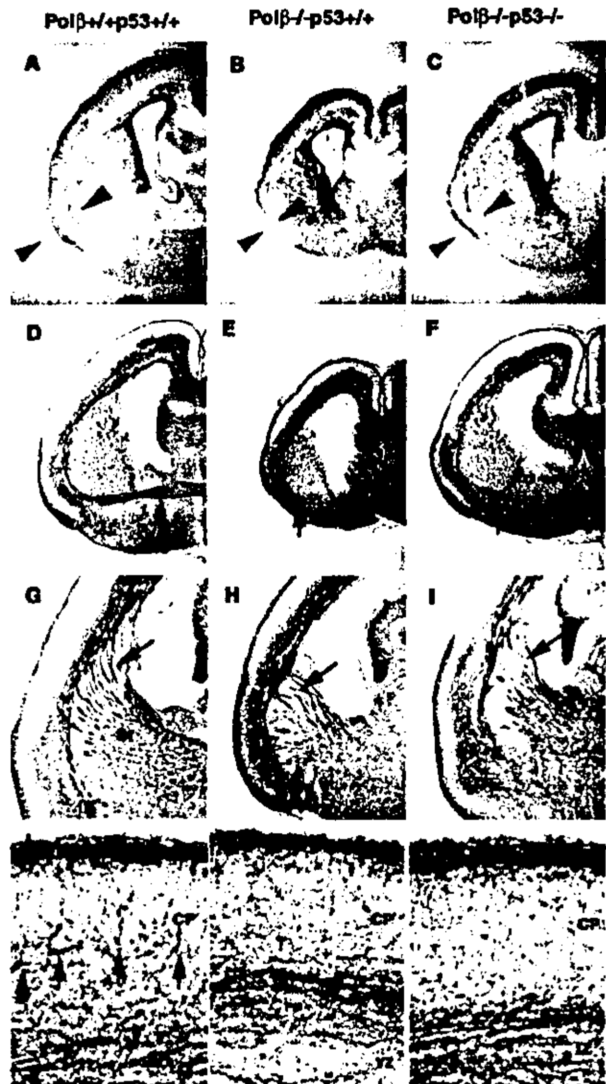

FIG. 5. Development of the brain is incomplete in E18.5 Polβ$^{-/-}$ p53$^{-/-}$ embryos. Coronal sections of telencephalons in Polβ$^{+/+}$ p53$^{+/+}$ (wild-type) (A, D, G, and J), Polβ$^{-/-}$ p53$^{+/+}$ (B, E, H, and K), and Polβ$^{-/-}$p53$^{-/-}$ (C, F, I, and L) embryos at E18.5 were stained with cresyl violet (A to C), anti-phosphorylated neurofilament antibody SMI31 (D to I), and anticalbindin antibody (J to L). Photographs of the sections in each genotype were taken with the same magnification. The arrowheads in panels A, B, and C indicate part of the cytoarchitecture recovered moderately in Polβ$^{-/-}$ p53$^{-/-}$ mice compared to Polβ$^{-/-}$ p53$^{+/+}$. The arrows in panels D, E, and F indicate the anterior commissure (AC). The arrows in panels G, H, and I indicate aberrant axonal tracts in the striatum (St). The arrows in panel J indicate calbindin-positive cells. MZ, marginal zone.

veloping neocortex are known to express calbindin D28k (Calbindin), an intracellular calcium-binding protein (2, 32). In Polβ$^{-/-}$ p53$^{+/+}$ embryos (Fig. 5K), we found a significant decrease in the number of calbindin-positive cells in the CP compared to wild-type embryos (Fig. 5J). More importantly, this decrease was not rescued by p53 deficiency in Polβ$^{-/-}$ p53$^{-/-}$ embryos (Fig. 5L). Taken together, these data, shown in Fig. 5, indicate that p53 deficiency does not completely rescue developmental defects in the central nervous system, associated with Polβ deficiency. These phenotypes are in sharp contrast to those of Lig4$^{-/-}$ p53$^{-/-}$ and XRCC4$^{-/-}$ p53$^{-/-}$ mice, which can be alive for several weeks after birth. These results suggest a crucial role for Polβ in the formation of the

intact neuronal circuit and migration of certain neuronal cell types.

DISCUSSION

We have shown here that p53 deficiency rescues neuronal apoptosis in Polβ-deficient mice and that haploinsufficiency substantially does so as well (Fig. 3). We have also shown that p53 is activated by its serine-18 phosphorylation in developing telencephalons of Polβ-deficient mice (Fig. 2). These results indicate that the neuronal apoptosis associated with Polβ deficiency is mediated by the p53-dependent pathway. In addition, it should be noted that, like Polβ$^{-/-}$ p53$^{+/+}$ neonates, Polβ$^{-/-}$ p53$^{-/-}$ neonates die shortly after birth.

The onset of p53-dependent repair or apoptosis is determined by the level of accumulated damaged DNA (36). There is ample biochemical evidence for functioning of Polβ mainly in the short-patch BER pathway to repair SSBs, which are mostly generated as intermediates of damaged bases metabolized by DNA glycosylase and AP endonuclease (46). Mouse embryonic fibroblasts defective in Polβ are highly sensitive to DNA alkylating agents but not to X-ray radiation (41, 42), supporting in vivo that Polβ deficiency leads to defects in SSB repair but not in DSB repair. Thus, Polβ deficiency should result in increased levels of SSBs, even if the Polβ-independent long-patch BER is able to partially substitute for the short-patch BER. The increased SSB levels would stabilize and activate p53, leading to apoptosis during neuronal differentiation in Polβ$^{-/-}$ mice. In mice defective in Lig4, XRCC4, Ku70/80, or XRCC2, which all function in DSB repair, differentiating neurons undergo massive apoptosis (4, 11, 14, 17, 22). Therefore, in these mice, unrepaired DSBs are thought to be the cause of the apoptosis (4, 11, 14, 17, 22). The apoptosis in Lig4$^{-/-}$ and XRCC4$^{-/-}$ embryos is rescued by p53 deficiency (15, 16). Lig4$^{-/-}$ p53$^{-/-}$ and XRCC4$^{-/-}$ p53$^{-/-}$ neonates can survive several weeks after birth without behavioral or neurological abnormalities. This is in sharp contrast with our observation that Polβ$^{-/-}$ p53$^{-/-}$ neonates die shortly after birth. The degree and quality of rescue by p53 deficiency in repair-deficient mice appear to vary depending on the type and level of DNA damage. As discussed above, in Polβ$^{-/-}$ mice, the damage is most likely SSBs, but the possibility that these SSBs are subsequently converted into DSBs in the final DNA replication of neuronal progenitor cells cannot be ruled out.

In Polβ$^{-/-}$ p53$^{-/-}$ mice at E13.5, early steps of neuronal differentiation seem to proceed normally, as judged by immunohistochemical analysis (Fig. 4). However, at E18.5, these and Polβ$^{-/-}$ p53$^{+/+}$ mice displayed serious cytoarchitectural defects in the major axonal tract (Fig. 5E and F) (with more aberrant axonal tracts in the striatum [Fig. 5H and I]) and the migration in GABAergic interneurons (Fig. 5K and L). These results suggest that, although p53 deficiency indeed rescues neuronal apoptosis, these neurons are still incomplete as mature ones, implying that the deficiency cannot fully restore the neuronal development of at least certain cell types. The brain is composed of remarkably complex neuronal cell types and networks. In the development of the brain, cell migration, axon growth, and pathfinding are fundamental processes (12). Recent studies with knockout mice have identified a number of molecules responsible for such processes (30, 32). Loss of these molecules severely affects the brain development and is critical for survival. The abnormal development of the nervous system observed in both Polβ$^{-/-}$ p53$^{-/-}$ and Polβ$^{-/-}$ p53$^{+/+}$ mice at E18.5 may be responsible for death shortly after birth. In Lig4$^{-/-}$ p53$^{-/-}$ or XRCC4$^{-/-}$ p53$^{-/-}$ mice, severe defects in lymphogenesis are never recovered by p53 deficiency, implying that Lig4 or XRCC4 is a critical factor for lymphogenesis (15, 16). Similarly, our finding that the neuronal differentiation in Polβ$^{-/-}$ mice is not completely rescued by p53 deficiency strongly suggests that Polβ is a critical factor for neurogenesis; that is, Polβ may absolutely be required for neuronal differentiation.

The reason why Polβ is required for neuronal differentiation remains obscure. One possibility is that in neuronal differentiation, a large amount of damaged bases and SSBs are generated by reactive oxygen species, which might occur particularly in some neuronal cell types actively undergoing migration and/or axon pathfinding. Recently, the Polβ-dependent pathway was shown to be induced in response to oxidative base damage (7). Polβ might specifically be required to repair those damaged bases and SSBs. Thus, Polβ deficiency would lead to increased levels of DNA damage and activation of p53, eventually resulting in apoptosis. A second possibility is that Polβ is involved in chromatin remodeling and transcription in neuronal differentiation. When neuronal progenitor cells become postmitotic neurons, they exit cell cycle and drastically alter the pattern of gene expression from immature to mature neurons (12). Transcriptional activation of a gene involves recruitment of not only a sequence-specific DNA-binding protein but also a coactivator complex, including proteins with chromatin-modifying activity. For example, DNA topoisomerase IIβ alters DNA topology and forms complexes with proteins involved in chromatin remodeling and transcription (25, 44). The enzyme-deficient mice show defects in the laminar organization of the neocortex and motor axon growth, resulting in a breathing impairment and death of the pups shortly after birth (31, 49). This finding suggests that the control of chromatin reorganization is indispensable for neuronal differentiation. Interestingly, we note that transcriptional coactivator p300 forms a physical and functional interaction with Polβ (23). p300 integrates a diverse signaling pathway for a number of sequence-specific transcription factors and activates transcription through chromatin remodeling via intrinsic histone acetyltransferase activity (20). Therefore, in association with p300 or related proteins, Polβ might function to maintain the integrity of genes being, or to be, expressed in certain neuronal cell types. A third possibility is that during neuronal differentiation, a genomic rearrangement factor(s) is expressed and generates a certain type of DNA damage (repairable by Polβ) to initiate a specific differentiation. In the immune system, the molecular mechanism of diversity by rearrangement of the immunoglobulin or T-cell receptor gene clusters is well understood (43). In V(D)J recombination, the lymphocyte-specific endonucleases RAG1 and RAG2 initially cleave specific recognition sequences in immunoglobulin loci, followed by completion of rearrangements through DSB repair by the action of nonhomologous-end-joining factors (18). Similarly, in the nervous system, neuronal diversity might be created by such genomic rearrangement (9, 48). If this is the case, DNA repair by Polβ would be an essential part of the diversity mechanism.

In conclusion, our studies show that p53 deficiency dramatically rescues neuronal apoptosis associated with Polβ deficiency, indicating that p53 mediates the apoptotic process in the nervous system. However, p53 deficiency cannot restore complete differentiation of neuronal progenitors and leads to lethality shortly after birth. These observations suggest a crucial role for Polβ in differentiation of specific neuronal cell types. In addition, it is evident that in neuronal differentiation, p53 acts as a gatekeeper to maintain genomic stability against various types of DNA damage (27). Further studies will be needed to elucidate the precise role of Polβ in neurogenesis.

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