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Original Research

Differentiation of Neural Cells in the Fetal Cerebral Cortex of Cynomolgus Monkeys (*Macaca fascicularis*)

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Proliferation and programmed cell death are important in the formation of morphologic structures and functional activity during CNS development. We used immunohistochemical and TUNEL methods to examine the proliferation and differentiation of neural cells in, distribution of apoptotic cells in, and microglial cell involvement in the removal of apoptotic cells from the fetal cerebral cortex of cynomolgus monkeys. At embryonic day (E) 50 and E80, the neuroepithelium contained many mitotic cells. Cells staining for PCNA (a nuclear marker of proliferating cells) were prominent in the proliferative zone, whereas cells positive for NeuN (a neuron-specific marker) were absent. GFAP staining for glial cells was positive in the neuroepithelium and radial glial fibers. Iba1-positive cells (that is, macrophages and microglia) were distributed throughout all regions at all time points but accumulated especially in the ventricular zone at E80. Apoptotic morphology (at E80) and TUNEL-positive cells (that is, containing DNA fragmentation; at E50 and E80) were observed also. At E120 and E150, most PCNA-positive cells were in the ventricular zone, and NeuN-positive cells were prominent in all layers except layer I-II at E120. GFAP immunoreactivity was detected mainly in cells with fine processes in the white matter. Neither apoptosis nor TUNEL-positive cells were detected at either E120 or E150. These results suggest that proliferation, migration, and neural cell death occur during midgestation (that is, E50 to E80) in fetal brain of cynomolgus macaques, whereas differentiation and maturation of neural cells occur after midgestation (E80).

Abbreviations: E, embryonic day; GW, gestational week; PCD, programmed cell death.

The proliferation and programmed cell death (PCD) of neural cells during development of the CNS are important in the formation of morphologic structures and functional activity of the brain.[4,26,35] In normal corticogenesis, neuronal and glial precursors proliferate in the neuroepithelium covering the ventricle, and postmitotic neuronal precursors migrate to the cortical plate as their final destination. During this process, radial glial cells guide migrating cells. Neuronal precursors that have completed migration differentiate into mature neurons to form cerebral cortical layers.[25] During cortical developmental processes, PCD—mainly apoptosis—emerges. PCD reportedly occurs during corticogenesis at early to mid-pregnancy in humans[1,6,26,31] but between embryogenesis and the postnatal period in rodents.[3,4] In addition, microglia infiltration emerges to remove cells that have undergone PCD.[10,26] Some reports, however, suggest that the distribution of microglia is not always correlated with PCD.[2,27] Therefore, microglial involvement in PCD has not been confirmed during fetal brain development. Moreover, PCD has often been described during cerebral development in humans and rodents, but few reports have examined PCD in monkeys.[13]

In the current study, we investigated the proliferation, differentiation, and distribution of apoptosis of neural cells in the fetal brain, especially the cerebral cortex, cynomolgus monkey at different developmental stages using histopathologic analysis, immunohistochemistry, and TUNEL method. In addition, we investigated the involvement of microglial infiltration in the removal of apoptotic cells from the fetal monkey brain.

Materials and Methods

Animals. Four pregnant cynomolgus monkeys (*Macaca fascicularis*) at gestational days 50, 80, 120, and 150 (n = 1 at each stage) were purchased from and maintained at Shin Nippon Biochemical Laboratories (Kagoshima City, Japan). Serologically normal monkeys that were imported from China and had passed quarantine were used in the present study. Animal breeding, mating, and operations were performed at Shin Nippon Biochemical Laboratories. In particular, female monkeys with normal menstrual cycles each were caged for 3 d with a healthy male monkey

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during the time of expected ovulation. After an observer confirmed copulation or intravaginal sperm, the second of the 3 mating days was defined as gestational day 0. All monkeys were housed according to ILAR guidelines in individual stainless steel cages (69 × 61 × 75 cm) at 26 ± 2 °C and 50% ± 10% humidity, on a 12:12-h light:dark cycle, and with 15 fresh-air changes hourly.[16] Each monkey received about 108 g of food pellets once daily and had free access to drinking water.

After normal pregnancies were confirmed by ultrasonography, fetuses were obtained by Caesarean surgery, were confirmed alive, were euthanized by pentobarbital through the umbilical vein, and underwent autopsy. Dams received ampicillin (Meiji Seika Pharma, Tokyo, Japan) and buprenorphine hydrochloride (Otsuka Pharmaceutical, Tokyo, Japan) intramuscularly for 3 d postoperatively, and the surgical site was disinfected daily for 1 wk after surgery. In the current study, fetal cerebra at embryonic day (E) E50, E80, E120, and E150 were fixed in 4% paraformaldehyde or Bouin solution and embedded in paraffin. Coronal sections (E80, E120, and E150) of the occipital lobe and sagittal sections (E50) of the whole brain were sliced at 2 µm and stained with hematoxylin and eosin for histopathologic examination.

This study was performed according to guidelines for animal experiments at Shin Nippon Biomedical Laboratories. All procedures and protocols were approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Immunohistochemistry. Immunostaining was performed by the labeled streptavidin–biotin method for rabbit polyclonal antibodies[12] and by the polymer-based method for mouse monoclonal antibodies.[29] Deparaffinized sections were treated with 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity in the tissue. After being washed in PBS, sections were autoclaved for 10 min at 121 °C to enhance immunoreactivity. The sections were incubated with Block Ace (DS pharma Biomedical, Osaka, Japan) for 1 h at room temperature, to prevent nonspecific binding of immunoglobulin. Tissue sections then were incubated overnight with primary antibodies against PCNA (proliferating cell nuclear antigen), a marker for cells in early G1 phase and S phase of the cell cycle (1:200; Dako, Glostrup, Denmark); NeuN, a marker of mature neurons (1:200; Chemicon International, Temecula, CA); GFAP (glial fibrillary acidic protein) a marker for neuroepithelium, radial glial fibers, and astroglia (1:1000; Dako); and Iba 1 (ionized calcium binding adapter molecule 1), a marker of macrophage and micloglia (1:1000; Wako, Osaka, Japan). For rabbit polyclonal antibodies, sections were incubated with biotinylated goat antirabbit IgG (1:500; Dako) for 30 min at 37 °C, followed by incubation with horseradish-peroxidase–conjugated streptavidin (1:500; Dako) for 30 min at room temperature. For mouse monoclonal antibodies, sections were incubated with EnVision+ (Dako) for 30 min at room temperature. Immunoreactivity was visualized by treating sections with 3, 3′-diaminobenzidine tetraoxide (Dojin Kagaku, Kumamoto, Japan). Sections were counterstained with hematoxylin or 0.1% methyl green.

Detection of apoptosis. Apoptosis was detected by TUNEL analysis (Apop Tag Peroxidase In Situ Apoptosis Detection Kit, Chemicon)[11] and immunohistochemistry using a rabbit polyclonal antibody against cleaved caspase 3 (1:50; Chemicon). TUNEL analysis was performed according to the manufacturer's protocol with minor modifications; for example, sections were pretreated with 1 µg/mL proteinase K (Wako) in PBS for 10 min at room temperature. Negative control sections were incubated with PBS as a substitute for terminal deoxynucleotidyl transferase. Caspase 3 immunohistochemistry was done as described earlier, except that Tris-buffered saline was substituted for PBS and a polymer-based detection method (EnVision+, Dako) was used.

Cell counts. TUNEL-, PCNA-, and NeuN-positive cells in each section at E50, E80, E120, and E150 were counted cells under light microscopy at 400× magnification. For E50 and E80, we focused on 3 layers: the cortical plate (including the marginal zone); intermediate zone; and proliferative zone (comprising the ventricular and subventricular zones). For E120 and E150, we evaluated 5 layers (namely, layers I-II, III, IV, V, and VI). After 5 random fields (1000 cells each) from each section were captured by using a CCD camera (Q Capture Pro; Nippon Roper, Tokyo, Japan), the number of positive cells per 1000 cells was computed by using Scion Image (Scion Corporation, Frederick, MD).

Results

Differentiation of neural cells at E50 and E80. Histologically, the cerebral wall included 5 areas: the ventricular zone, subventricular zone, intermediate zone, cortical plate, and marginal zone. At E80, the proliferative zone, which comprises the ventricular and subventricular zones, was slightly smaller and intermediate zone and cortical plate were larger than those of E50 (Figure 1). Mitoses were observed in the neuroepithelium at E50 and E80 (Figure 2 A and B). Apoptosis characterized by nuclear fragmentation, pyknosis, and phagocytosis of the apoptotic body by macrophages was observed mainly in proliferative zone at E80 (Figure 2 C through E).

By immunohistochemistry at E50 and E80, PCNA-positive cells were detected mainly in proliferative zone (Figure 3 A and B). PCNA-positive cell counts were 771.5 and 618 per 1000 cells at E50 and E80, respectively. However, few PCNA-positive cells were found in intermediate zone and cortical plate (Table 1). GFAP immunoreactivity was detected in the neuroepithelium of the proliferative zone (Figure 3 C and D). Radial glial fibers elongating vertically from the proliferative zone to cortical plate were strongly positive (Figure 3 E). Iba1 immunoreactivity was detected in all layers, particularly at E80, when many positive cells were localized in proliferative zone (Figure 3 F). In addition, some positive cells phagocytized cellular debris (Figure 2 F). NeuN immunoreactivity was not detected at either E50 or E80 (data not shown).

TUNEL-positive cells at E50 and E80 were sparsely distributed in each layer (Figure 2 G), except for the cortical plate at E50, when positive cells were not detected. Overall there were very few TUNEL-positive cells (0.6 to 2.1 per 1000 cells; Table 1). Caspase 3 immunoreactivity was not detected.

Differentiation of neural cells at E120 and E150. Histologically, the cerebral cortex at E120 and E150 consisted of 6 layers (Figure 4 A and B). No cells at either time point showed characteristics of apoptosis.

Immunohistochemically, PCNA-positive cells were detected mainly in the ventricular zone, with a few PCNA-positive cells in the white matter and cerebral cortex at E120 and E150. PCNA-positive cell counts in ventricular zone were 833.1 and 658.3 per 1000 cells at E120 and E150, respectively (Table 2). NeuN immunoreactivity was detected in layers III through VI at E120 and in all layers at E150 (Figure 4 C and D; Table 3). Moreover, NeuN-positive cells at E120 had scant cytoplasm (Figure 4 C

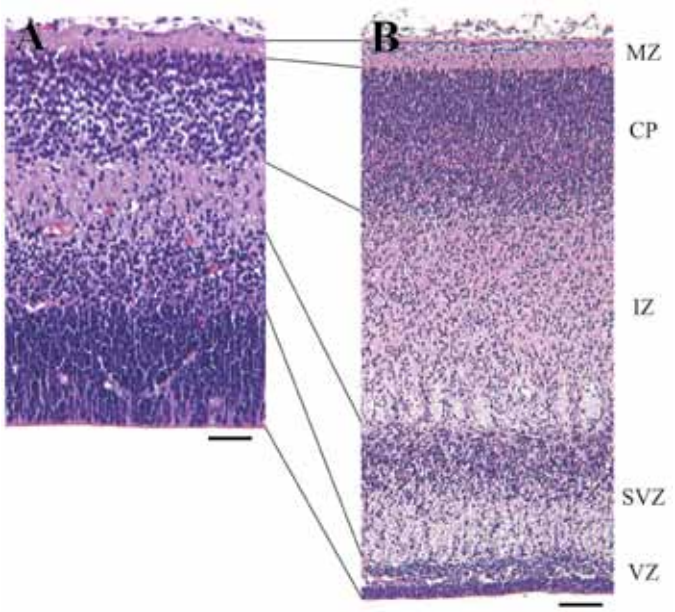

Figure 1. Cerebral wall at (A) E50 and (B) E80. The cerebral wall consisted of the marginal zone (MZ), cortical plate (CP), intermediate zone (IZ), subventricular zone (SVZ), and ventricular zone (VZ). Hematoxylin and eosin stain; bar, 50 µm.

inset), whereas at E150, some cells had abundant cytoplasm with processes resembling those of mature neurons (Figure 4 D inset). Radial glial fibers that were positive for GFAP were not present (Figure 4 E), the white matter contained many GFAP-positive cells with multiple processes (Figure 4 F) at E120 and E150. Iba1 immunoreactivity was present in each layer at E120 and E150. There were no TUNEL- or caspase 3-positive cells in any section at E120 or E150.

Discussion

This study investigated the proliferation, differentiation, and distribution of apoptosis of neural cells and the involvement of microglia in removal of apoptotic cells in the fetal cynomolgus monkey brain, especially the cerebral cortex, at different developmental stages. Because few studies have addressed the precise neural development of the cynomolgus monkey fetal brain, we mainly compared our results with those in rodents, humans, and rhesus monkeys (*Macaca mulatta*).

Proliferation and the migration of neural cells are known to be important in CNS development.[5] In our study, we noted a high frequency of mitoses in the ventricular zone at both E50 and E80. Using immunohistochemistry with PCNA to detect the proliferative activity of neural cells, we noted many positively stained cells in the proliferative and ventricular zones from E50 to E150. This finding of many mitotic cells in the ventricular zone is consistent with previous reports.[22,32] Moreover, the production of cortical neurons in the monkey visual area has been reported to start at E40 and finishes by E100.[25] Therefore, we speculate that cortical neurons actively proliferate between E50 and E80 and that this division and proliferation is complete by E120. Other reports show that numbers of PCNA-positive cells are significantly reduced when cell migration is active,[22,32] and these cited results are inconsistent with our finding that proliferative activity

Table 1. Number of PCNA- and TUNEL-positive cells per 1000 cells in each layer of the cerebral wall at E50 and E80

	E50		E80	
	PCNA	TUNEL	PCNA	TUNEL
Cortical plate	0	0	0.2	0.7
Intermediate zone	0.2	0.6	0.6	1.6
Proliferative zone	771.5	1.2	618	2.1

Table 2. Number of PCNA- and TUNEL-positive cells per 1000 cells in each layer of the cerebrum at E120 and E150

	E120		E150	
	PCNA	TUNEL	PCNA	TUNEL
Layers I through VI	10.4	0	10.3	0
White matter	77.9	0	42.5	0
Ventricular zone	833.1	0	658.3	0

Table 3. Number of NeuN-positive cells per 1000 cells in each layer of the cerebral cortex at E120 and E150

Layer	E120	E150
I-II	25.1	894.7
III	674.3	946.5
IV	673.6	947.7
V	557.2	929.5
VI	681.4	952.4

NeuN-positive cells were not detected at E50 and E80.

in the ventricular zone is maintained at a high level after E120. Whereas the previous studies addressed the human fetal brain,[22,32] we used the cynomolgus monkey fetus, and differences observed may reflect species-associated differences between humans and macaques in neural cell development during the final stage of CNS maturation.

We noted few PCNA-positive cells in the intermediate zone and cortical plate at E50 and E80. PCNA-positive cells increased in the cerebral cortex and medulla at E120 and E150, but frequencies were lower than those in the ventricular zone. Humans display a few PCNA-positive cells in intermediate zone.[22,32] In addition, the presence of PCNA-positive cells in the intermediate zone has suggested their role in the glial system.[17] In the rat developing brain, most microglial precursors show high proliferative activity,[8] and the proliferating cells in the cortical plate are thought to be glioblasts or angioblasts.[5] In light of these previous and our current findings, we speculate that PCNA-positive cells at E50 and E80 in areas other than the proliferative zone might be glial cells.

Apoptosis plays an important role in the developing CNS.[5,7] In human cerebral development, apoptosis is induced mainly in the proliferative zone during early pregnancy (11 gestational weeks [GW]),[26] increases in all regions of the telencephalon and from marginal zone to ventricular zone except for cortical plate during the second trimester of pregnancy,[26,31] and is detected in the cerebral cortex during late pregnancy.[26,33] In proliferative zone at E80 in the present study, we observed chromatin condensation and nuclear fragmentation, which are well-known morphologic characteristics of apoptosis. We noted only a few TUNEL-positive cells in each layer at E50 and E80 and no TUNEL-positive cells at all after E120. Moreover, there was little difference in temporal

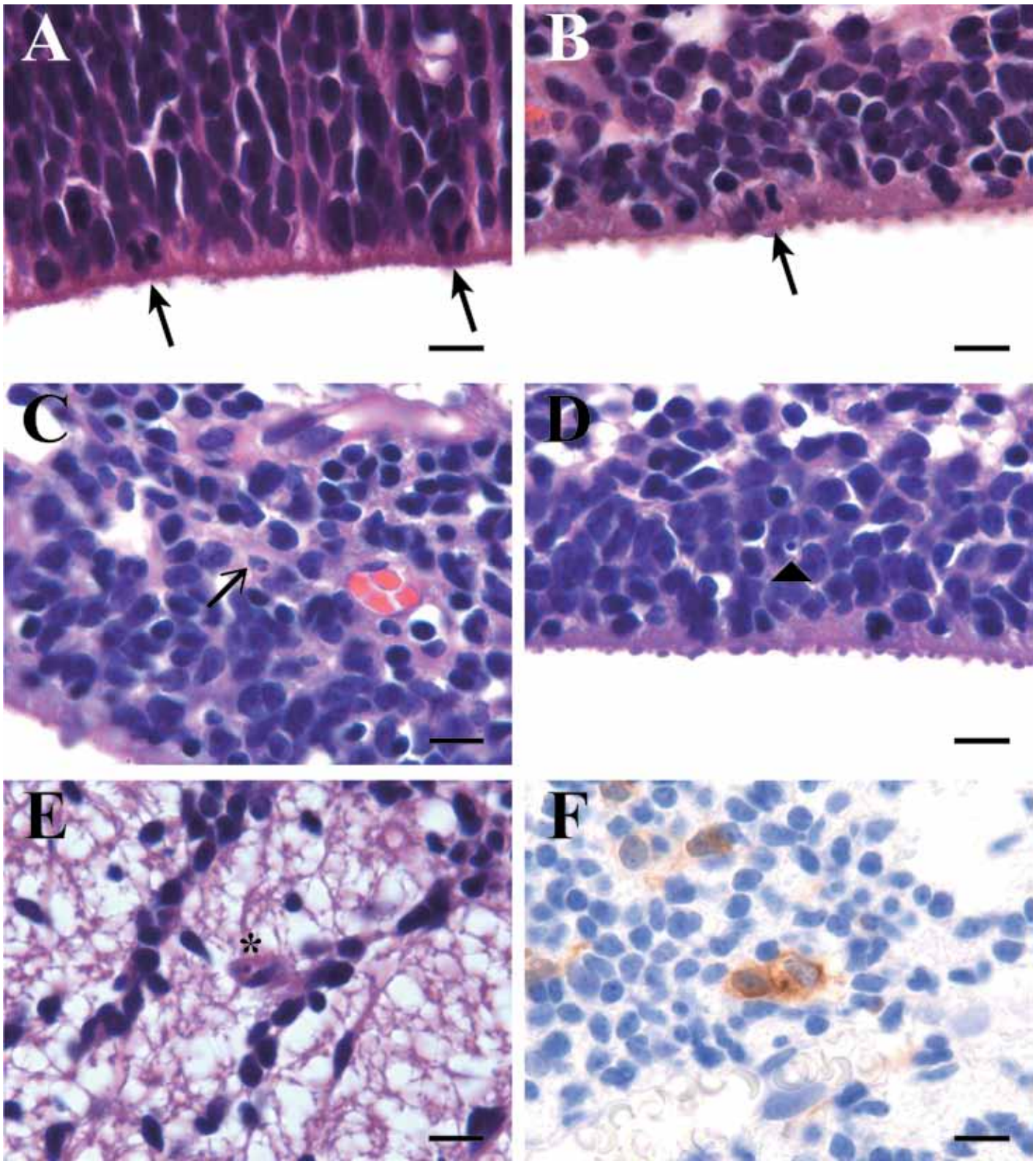

Figure 2. Ventricular zone at (A) E50 and (B through F) E80. (A, B) Mitoses (arrows) were noted in the bottom layer of the neuroepithelium. (C through E) Nuclear fragmentation (arrow), pyknosis (arrowhead), and macrophagic (asterisk) phagocytosis of cells with these characteristics were observed. (F) Cellular debris was phagocytized by Iba1-positive cells. (A through E) Hematoxylin and eosin stain; (F) Immnohistochemical staining of Iba1; bar, 10 μm.

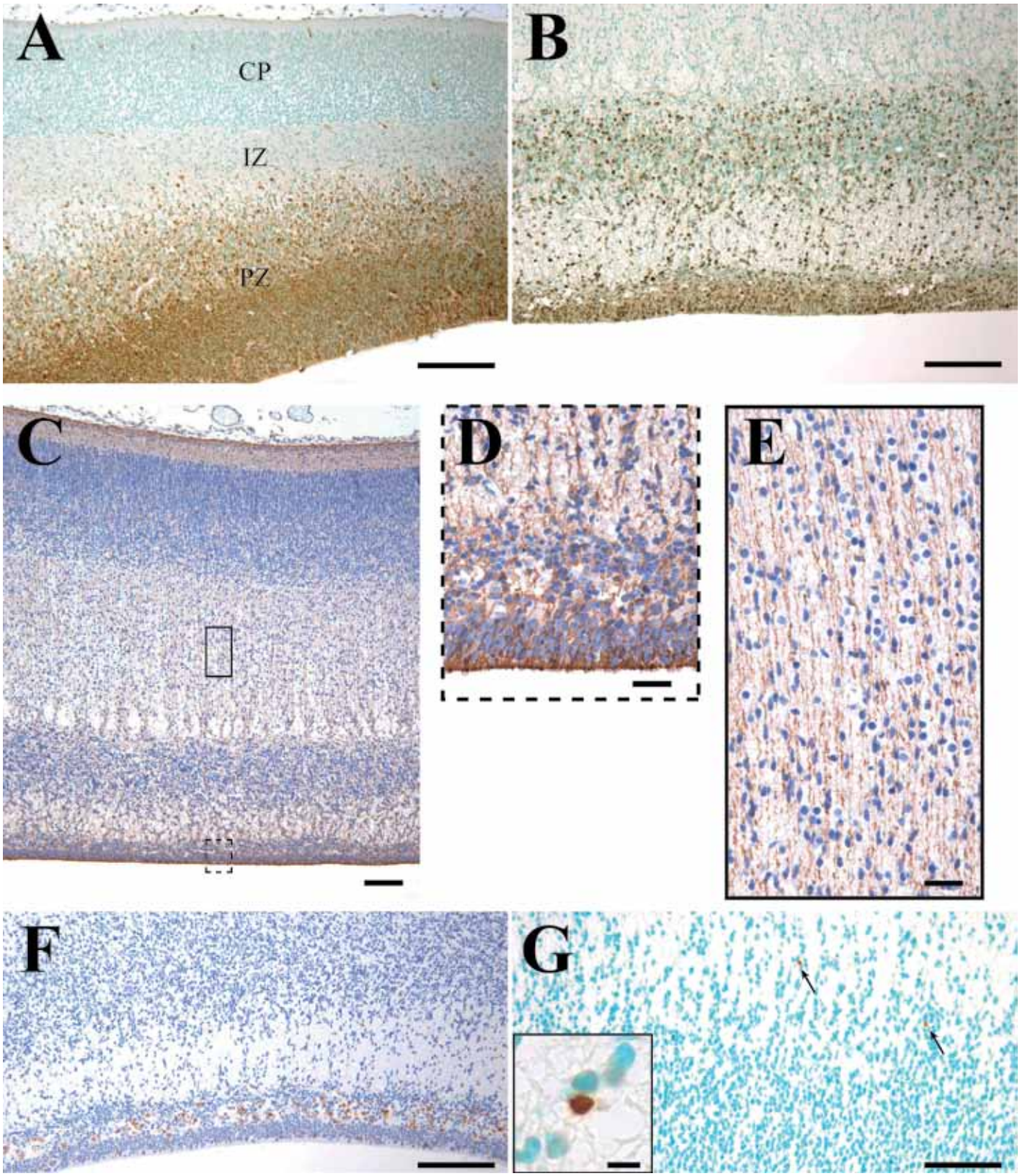

Figure 3. Immunohistochemical staining of PCNA at (A) E50 and (B) E80; methyl green counterstained. Most cells in the proliferative zone (PZ, ventricular and subventricular zones) were positive. (C through E) Immunohistochemical staining of GFAP and (F) Iba1 at E80; hematoxylin counterstained. (D) Neuroepithelium and (E) radial glial fibers that extend throughout the intermediate zone were positive for GFAP, whereas (F) Iba1-positive cells were localized in the ventricular zone. (G) TUNEL staining at E50; methyl green counterstained. TUNEL-positive cells (arrows) were sparsely distributed in the ventricular zone. Bar: 100 µm (A through C, F, and G); 25 µm (D, E); 2 µm (inset of G).

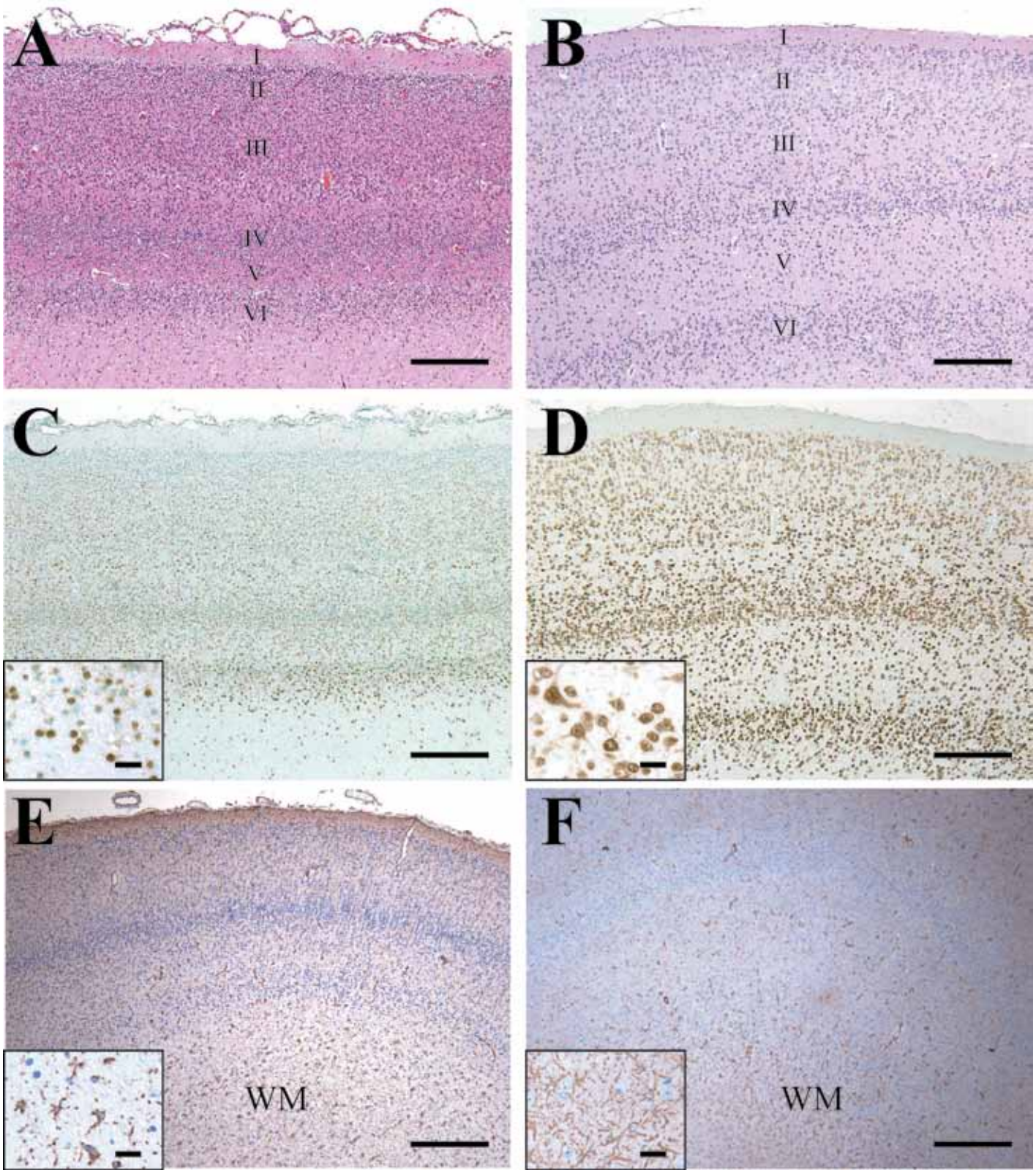

Figure 4. Hematoxylin and eosin staining of the cerebral cortex at (A) E120 and (B) E150. The cerebral cortex consisted of 6 layers. (C) Immunohistochemical staining of NeuN; methyl green counterstained. Most cells in all layers, except for layer I-II, were strongly positive. (C, inset) Higher magnification of layer III shows that the nuclei of cells with scant cytoplasm were positive. (D) Almost all cells of the cerebral cortex were NeuN-positive, with particularly strong staining both the nucleus and cytoplasm of cells with processes in layer III (inset). Immunohistochemical staining of GFAP at (E) E120 and (F) E150; hematoxylin counterstained. Many cells with short processes mainly in the white matter (WM, inset) were strongly positive. Radial glial fibers observed at E80 were not present (inset). Bar: 200 µm (E); 100 µm (A through D); 50 µm (F); 20 µm (inset of C through F).

and spatial patterns between E50 and E80. TUNEL-positive cells in the current study were fewer (0 to 2.1 per 1000 cells) than those reported for rodents,[3,4] humans,[26,31] and rhesus monkeys.[13] This result may be explained by the fact that apoptotic cells are removed rapidly, in about 2 to 3 h.[34]

Apoptosis is thought to induce timely disappearance of various structures during brain development. For example, in humans, TUNEL-positive cells are detected in the marginal zone beginning during the second trimester of pregnancy. The numbers of apoptotic cells increase until the disappearance of the subpial granular layer (23 to 30 GW) and significantly decrease overall in layer I after 30 GW.[33] However, in the current study, the TUNEL method failed to detect any apoptotic cells in the cerebral cortex after E120. Therefore, apoptosis-induced temporal disappearance of brain structure may not occur after this point in fetal cynomolgus macaques. In addition, we noted very few apoptotic cells at or before E80, when the proliferation and migration of neural cells were active, indicating that apoptosis plays an important role in targeting undifferentiated or irregularly differentiated cells.[3]

To investigate the involvement of microglia in the removal of apoptotic cells, we performed immunohistochemistry using antiIba1 antibody. Iba1 is a 17-kDa EF-hand protein that is specifically expressed in macrophages and microglia and is upregulated during the activation of these cells.[15] In the current study, Iba1 immunoreactivity was detected during gestation, but it was not temporally correlated with apoptotic cells, a finding that is inconsistent with previous reports from rat and human fetal brains.[10,26] Although we noted no temporal correlation between microglial accumulation and apoptosis, other studies report the aggregation of microglial cells around the neuroepithelium in the telencephalic wall that contain phagocytized pyknotic nuclei, suggesting the involvement of these microglia in cell death.[2,24] In addition, the present study revealed the presence of Iba1-positive cells (that is, microglial cells) that had phagocytized cellular debris in proliferative zone at E80. As mentioned earlier, microglia may be associated with the removal of dead cells in the developing brain, because characteristic features such as nuclear fragmentation were present in the proliferative zone at E80. A characteristic finding in the present study was the aggregation of microglia localized around the ventricular zone at E80. This distribution at E80 corresponds to that of humans between 12 to 24 GW, which is equivalent to E50 to E80.[28] The aggregated microglial cells may be involved with the disappearance of temporary structures in the developing brain, such as the subventricular and subplate zones, because the subventricular zone declines dramatically between mid- and late gestation.[5] Alternatively, microglial cells may enter the CNS from within or around the ventricular and subventricular zones during CNS development. In either case, the cause of aggregation remains unclear, and further studies are needed to define the functional roles of microglia during brain development.

To investigate the differentiation of neuronal cells, we performed immunohistochemistry using antiNeuN antibody. NeuN is a transcriptional factor that is expressed in the nucleus and cytoplasm of neurons after postmitotic precursor cells start to differentiate into mature cells cytologically and morphologically.[21] Moreover, immature neuronal cells that do not have mature function are negative for NeuN. Therefore, NeuN is a useful marker of the developing CNS because it labels the nucleus rather than the cytoplasm and can be detected even in cells with scant cytoplasm.[30,36] In the current study, NeuN immunoreactivity was not detected at E50 or E80, whereas most cells in the cerebral cortex were positive at E120 and E150. In humans, NeuN is expressed in layers IV to VI at 20 GW,[30] although some reports show that expression of NeuN is not revealed at 26 GW.[1] The visual cortex during E70 to E105 in monkeys is histologically similar to that during 18 to 30 GW in humans,[18] and data obtained from the present study were consistent with those in humans. In addition, laminar formation of the cerebral cortex is induced during approximately this same time frame.[18] Therefore, these combined data indicate that functional neuronal differentiation starts around the time of laminar formation of the cerebral cortex.

Radial glial cells are important in guiding the migration of immature neurons, which differentiate into astrocytes after completing migration.[25] Some reports show that they also differentiate into neurons.[20,23] GFAP immunohistochemistry in the current study revealed the existence of glial cells with varied morphology. At E50 and E80, the neuroepithelium was positive for GFAP. Radial glial fibers elongating vertically from the ventricle to pia mater between E50 and E80 were strongly positive, but the numbers of radial glial cells had decreased by E120, and cells with short processes, such as mature astrocytes, increased. These results are reasonably consistent with a previous report in rhesus monkeys.[19] Some reports in humans show that radial glial cells decrease beginning in the second trimester, and mature astrocytes appear at 30 GW.[9,14,33] These data closely reflect the findings of the present study.

In conclusion, the cerebrum of fetal cynomolgus monkeys at E50 and E80 contained many mitoses and PCNA-positive cells, as well as radial glial fibers that help in cell migration. Apoptosis, phagocytosis, and aggregated microglia were present. These results suggest that proliferation, migration, and cell death of neural cells are predominant until mid-gestation (E80). At E120 and E150, the cerebral cortex showed a decrease in the number of proliferating cells, the disappearance of radial glial fibers, and the appearance of mature astrocytes and mature neurons. These results suggest that differentiation or maturation of neural cells starts after the mid-gestational period in the cynomolgus monkey fetus. These findings are relevant to the timing of neurologic developmental events in nonhuman primates and, because of the physiologic similarities of humans to nonhuman primates, are pertinent to the use of nonhuman primates for neurodevelopmental toxicologic research

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