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Cell cycle and cell death regulation of neural progenitor cells in the 5-azacytidine (5AzC)-treated developing fetal brain

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Abstract

In the developing brain, neural progenitor cells are susceptible to many extrinsic stresses, including DNA damage. We treated pregnant rats with 5-azacytidine (5AzC), a DNA demethylating and damaging agent, to investigate the cellular responses of the fetal brain, focusing on the regulation of proliferation and cell death. 5AzC first induced the accumulation of cells in abnormal mitosis, G2-phase accumulation, and then apoptosis of the neural progenitor cells. Most of the apoptotic cells were in G1 phase. Cell cycle transition studies suggested that G2/M progression was blocked, after which the cells moved to G1 phase or underwent apoptosis. p53, a key factor for response to DNA damage, and some of its target genes showed increased expression in Western blot and DNA microarray analyses. In 5AzC-treated fetal brains of p53-deficient mice, apoptosis did not occur, although G2/M accumulation was induced. These results suggest that, in the developing brain, apoptosis is p53-dependent but that another mechanism governs the G2/M checkpoint. The G2/M regulator, Cdc2, was activated by dephosphorylation through G2/M accumulation, suggesting accelerated entry into mitosis leading to accumulation of cells showing abnormal mitosis. Furthermore, some cells may have died due to mitotic catastrophe. Throughout brain development, various cell cycle and cell death regulation mechanisms provide neural progenitor cells with options for defense from DNA damage.

Keywords: Apoptosis; 5-azacytidine; Cell cycle arrest; DNA damage; Interkinetic nuclear migration; Mitotic catastrophe; Neural progenitor cell; p53; Ventricular zone

Introduction

Environmental stresses and stimuli can induce deleterious effects on brain development. The fetal central nervous system (CNS) is sensitive to diverse environmental factors because a large number of processes occur during an extended period of development, and fetal neural damage is an important issue affecting the completion of normal CNS development (Rodier, 1995; Mendola et al., 2002; Costa et al., 2004).

In the developing brain, multipotent neural progenitor cells proliferate in the ventricular zone (VZ), after which they differentiate into neural cells, i.e., neurons, astrocytes, and oligodendrocytes (Rao, 1999; Qian et al., 2000; Temple, 2001). In the early developmental stage, they form a pseudostratified epithelium in the VZ, so they also are called neuroepithelial cells. The nuclei of proliferating neural progenitor cells undergo a characteristic migration–interkinetic nuclear migration (or "elevator movement")–in the VZ, in which the positions of nuclei are correlated with their cell cycle phase (Takahashi et al., 1995; Fujita, 2003). In brief, the S phase nuclei located in the outer area of the VZ translocate inward during G2 phase, and mitosis occurs at the ventricular surface. Then, the nuclei migrate outward during G1 and enter S phase again (Fig. 7A). In this way, neural progenitor cells proliferate.

The balance between proliferation and cell death (apoptosis) is important for correct development of the brain (Oppenheim, 1991; Blaschke et al., 1996; Thomaidou et al., 1997). Moreover, the regulation of this balance also seems to be important during damage due to extrinsic stresses, particularly that from anti-proliferative stimuli. However, it remains unclear how neural progenitor cells in the fetal brain react toward extrinsic stresses, especially regarding the regulation of proliferation and cell death, while the organ is still developing.

5-Azacytidine (5AzC) is an agent that has two characteristic effects that interfere with the brain development, i.e. disturbance

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of DNA methylation and DNA damage. During the development of the CNS as well as other organ systems, DNA methylation is a key step for regulating gene expression (Sun et al., 2003), and agents such as 5AzC may disturb gene expression, and subsequently organogenesis, through their DNA demethylating effects. 5AzC also is thought to act as a DNA damaging agent (Juttermann et al., 1994; Karpf et al., 2001), and DNA damage causes serious abnormalities in the developing brain (Gao et al., 1998; Vinson and Hales, 2002; D'Sa et al., 2003). In our previous study, we demonstrated that 5AzC treatment of the pregnant rats prompted neural progenitor cells in the fetal brain to undergo apoptotic cell death; the treatment also led to delayed migration of nuclei, suggesting that cell cycle arrest might occur (Ueno et al., 2002a,b). In addition, our data indicated that these events might be dependent on p53, a tumor suppressor protein, and its transcriptional target genes such as $p21^{waf1/cip1}$. p53 is known to play a key role in the induction of cell growth arrest and apoptosis in response to DNA damage (May and May, 1999; Lakin and Jackson, 1999). However, despite these observations, it was unclear at which cell cycle phase the neural progenitor cells arrested and underwent apoptosis due to 5AzC treatment.

In the present study, we exposed fetal rat brains to 5AzC to examine how the neural progenitor cells response to extrinsic stresses, focusing on the regulation of apoptosis and cell cycle kinetics. We used flow cytometric methods for investigating alterations in cell cycle distribution and assessing the cell cycle position of apoptotic cells. Furthermore, we evaluated alteration of gene expression by using DNA microarray technology to reveal various mechanisms underlying cellular responses to 5AzC. In our examination, we focused on the role of p53 in the regulation of apoptosis and the cell cycle, and we used p53-knockout mice to confirm these points. We found that both p53-dependent and -independent mechanisms were involved in the regulation of apoptosis and the cell cycle of 5AzC-treated neural progenitor cells.

Materials and methods

All procedures were approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Animals

Pregnant Jcl:Wistar rats were obtained from Japan CLEA (Tokyo, Japan). $p53^{+/-}$ mice (C57BL/6 TSG-p53 N5 Targeted Mutation) were purchased from Taconic (Germantown, NY). Heterozygous mice were crossed to generate wild-type, heterozygous, and homozygous gene-disrupted mice. Endogenous and disrupted genes were detected by polymerase chain reaction analysis of tail DNA extracts, as described by Timme and Thompson (1994).

Chemicals

5AzC and 5-bromo-2′-deoxyuridine (BrdU) were obtained from Sigma (St. Louis, MO).

Treatments for Wistar rats

On day 13 of gestation pregnant, rats were injected intraperitoneally (i.p.) with 10 mg/kg of 5AzC and then euthanized at 1, 3, 6, 9, and 12 h after treatment. The dose was selected according to that of a previous study, in which 10 mg/kg of 5AzC caused high induction of neural cell apoptosis and low fetal mortality (Lu et al., 1998). As controls, pregnant rats were injected with an equivalent volume of saline and euthanized at 1, 3, 6, 9, and 12 h after treatment. Collected fetuses underwent histopathological examination, and cell cycle, DNA microarray, and Western blot analyses.

Treatments for p53-knockout mice

Pregnant mice were injected i.p. with 10 mg/kg of 5AzC on day 12 of gestation and euthanized at 6 and 12 h after treatment. As controls, dams were injected i.p. with an equivalent volume of saline on day 12 of gestation and were euthanized at 6 h after treatment. Collected fetuses were subjected to histopathological examination and cell cycle analysis.

Histopathological examination and immunohistochemistry

Collected fetuses were fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections (thickness, 4 μm) were stained with hematoxylin and eosin for histopathological examination. The dorsal telencephalic wall was mainly examined.

Some of the sections underwent immunohistochemical staining for cleaved caspase-3 and phospho-histone H3 by the LSAB method with streptavidin (Dako, Carpinteria, CA). Rabbit anti-cleaved caspase-3 polyclonal antibody (Cell Signaling Technology, Beverly, MA) and rabbit anti-phospho-histone H3 polyclonal antibody (Cell Signaling Technology) were used as the primary antibodies and biotin-labeled goat anti-rabbit IgG (Kirkegaard and Perry, Gaitherburg, MD) as the secondary antibody.

Staining for p53 and $p21^{waf1/cip1}$ was performed with an Envision+ Kit (Dako), as previously reported (Ueno et al., 2002a). Rabbit anti-p53 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-p21 monoclonal antibody (Pharmingen, San Diego, CA) were used as the primary antibodies. The positive signals were visualized using a peroxidase-diaminobenzidine reaction, and then the sections were counterstained with methyl green.

Cell cycle analysis

Telencephalons of two or three fetuses from each dam (1 to 12 h after treatment, and controls) were obtained carefully under stereoscopic microscopy and then prepared for flow cytometric analysis. The cells isolated from the telencephalons from each dam were resuspended in HBSS. The concentration of the resuspended cells was adjusted to $1–2 \times 10^6$. They were centrifuged for 5 min at $1500 \times g$ at 4°C, and the supernatant was discarded. After being washed in Dulbecco's PBS (dPBS), the cells were fixed in 70% ethanol at 4°C overnight. Cells then