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Possible involvement of DNA methylation in 5-azacytidine-induced neuronal cell apoptosis

H. Nakayama, S. Kajikawa, J. Shinozuka, W-P. Su and K. Doi

Department of Veterinary Pathology, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

Summary. Eight chemicals that are cytidine analogues or nucleosides (5-azacytidine (5AzC), 5-azadeoxycytidine, 6-azacytidine, 5-azacytosin, cytidine, 3-deazaadenine, 3-deazauridine and 6-azauridine) were examined for the ability to induce neuronal apoptosis. 5AzC and 5-azadeoxycytidine induced apoptosis in the brain and spinal cord of the fetuses at 24 hr after the injection to dams, while the other chemicals tested failed to induce apoptosis. In the system of PC12 cells, only 5AzC induced apoptosis, and other chemicals failed to provoke morphological and biochemical changes characteristic of apoptosis. 5AzC, 5-azadeoxycytidine and 6-azacytidine failed to induce apoptosis in C6 cells. Gel electrophoresis after MspI or HapII digestions revealed no apparent evidence of DNA demethylation after 5AzC-treatment in either fetal brains or PC12 cells. These results indicate that DNA demethylation is possibly involved in 5AzC-induced neuronal apoptosis although no direct evidence of DNA demethylation was obtained.

Key words: Apoptosis, 5-Azacytidine, DNA methylation, Brain, PC12 cells

Introduction

5-Azacytidine (5AzC) is a cytidine analogue which possesses nitrogen atom instead of carbon atom at the 5 position of the pyrimidine ring. 5AzC is known to induce cell differentiation in some murine embryonal cell lines (Constantinides et al., 1977, 1978; Jones and Taylor, 1980; Jones, 1985; Jüttermann et al., 1994) and cell death in some human leukemia cell lines (Pinto and Zagonel, 1993; Murakami et al., 1995) and some mammalian cells lines (Davidson et al., 1992). These events are assumed to be due to DNA demethylating function of 5AzC (Jones and Taylor, 1980; Creusot and Christman, 1981; Creusot et al., 1982; Jones, 1985; Iguchi and Schaffner, 1989). There are also several reports that 5AzC causes brain cell death in the fetuses from the dams injected with the chemical (Takeuchi et al., 1977; Takeuchi and Murakami, 1978; Takeuchi and Takeuchi, 1978; Schmahl, 1979; Schmahl et al., 1984). We have clarified that such brain cell death occurs in the form of apoptosis (Hossain et al., 1995). 5AzC-induced apoptosis also takes place in the murine primary fetal brain cell culture (Hossain et al., 1996) and in the rat pheochromocytoma-derived PC12 cell line (Hossain et al., 1997a,b). Accordingly, we speculated that 5AzC-induced neuronal cell apoptosis would be due to an activation of a certain apoptosis-related gene through DNA demethylation. Our previous *in vivo* observations revealed that 5AzC-induced apoptosis mainly occurred in the migratory to cortical zones of the brain wall which are composed of cells just after division (Hossain et al., 1995). In addition, it was clarified that 5AzC could induce apoptosis in the actively dividing PC12 cells without nerve growth factor (NGF)-treatment which induces differentiation of PC12 cells. These findings will support the above-mentioned speculation because 5AzC would be taken into DNA and would induce cytosine demethylation during and/or just after cell division.

In order to prove our speculation, we tried to investigate the relation between the induction of apoptosis and the state of DNA methylation in the present study. The present results indicated the involvement of DNA demethylation during 5AzC-induced neuronal apoptosis although no direct evidence of DNA demethylation was obtained.

Offprint requests to: Dr. H. Nakayama, Department of Veterinary Pathology, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yoyoi, Bunkyo-ku, Tokyo 113-8657, Japan

Materials and methods

Chemicals

5-azacytidine (5AzC), 5-azadeoxycytidine (5AzdeC), 6-azacytidine (6AzC), 5-azacytosin (5Azcytosine), cytidine, 3-deazaadenine (3deAzA), 3-deazauridine (3deAzU) and 6-azauridine (6AzU) were perchased from Sigma, St. Louis, MO.

Animals

Pregnant ICR female mice were obtained from Saitama Laboratory Animal Company (Saitama, Japan). Mice were injected intraperitoneally with 0.4 mg of the chemicals resolved in phosphate-buffered saline (PBS) or with PBS alone as control at 11th day of gestation. They were euthanized by ether inhalation at 1 to 24 hr after the chemical injection. Fetuses were removed and then processed for further experiment or fixed in 10% neutral-buffered formalin.

Histopathology

Fixed samples were embedded in paraffin and 4 to 6 μm-thick sagittal sections were made. The sections were stained with hematoxylin and eosin (HE) and some selected ones were applied to a modified TUNEL stain (Gavrieli et al., 1992) for detecting apoptotic cells *in situ* (ApopTag^{TM}, Oncor, Gaithersburg, MD, USA).

Cell cultures

PC 12 cells derived from a rat pheochromocytoma and C6 cells from a rat glioma were used for *in vitro* induction of apoptosis. PC 12 cells were cultured in high-glucose Dulbeco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS, JRH Biosciences, Australia) and 5% horse serum (JRH Biosciences). C6 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. One mg of each chemical was added into the cell cultures including approximately 10^7 cells. The cells were collected at 1, 3, 6, 12 and 24 hr after adding the chemicals.

Detection of apoptosis by immunoabsorbant assay

The Cell Death Detection ELISA kit (Böhringer-Mannheim, Germany) was used to detect apoptotic cell death of the cultured cells. Cells were lysed in the incubation solution included in the kit at 4 °C for 30 min. Lysate was centrifuged at 20,000g for 10 min. The supernatant, including lower molecular DNA, was collected. Appropriate dilutions of the supernatant were put into the wells coated with an anti-histon antibody. After incubation at room temperature for 90 min, wells were washed with the washing solution included in the kit. Then, the peroxidase-labeled anti-DNA antibody was put into the wells and the wells were incubated at room temperature for 90 min. Color development was done by adding a substrate solution including 2,2'-azino-di-[3-ethylbenzothiazoline sulfonate] (ABTS). The optical density (OD) of the sample solutions was measured at 405 nm wave length with a spectrophotometer. Each value was obtained by subtracting OD of the blank background from OD of the sample and the final results were expressed as the ratio to OD of the living cell control.

DNA methylation assay

Total genomic DNA was extracted from fetal brains or cell cultures using a DNA extraction kit (QIAmp Tissue kit, QIAGEN, Germany) and digested with endonucleases, MspI or HapII (Takara, Shiga, Japan). These two enzymes are known to recognize the same sequence site (CCGG). However, when the cytosine base is methylated, the former can cut DNA at the site but the latter cannot. Digested DNA was electrophoresed on 0.8% agarose gel (Seaken GTG, FMC BioProducts, Rockland, ME) and the gel was stained with ethidium bromide.

Results

Induction of apoptosis in fetal mouse neuronal tissues and PC12 cells

There was no change in the fetal mouse brain and spinal cord up to 6 hr after the injection of 5AzC (Fig. 1a-d). A few cells in the migratory zone of the brain wall and in the mantle layer of the spinal cord indicated pyknotic and/or karyorrhectic nuclei at 12 hr after the injection (Fig. 1e). Most cells in the layers were affected with such nuclear changes at 24 hr (Fig. 1f). The pyknotic or karyorrhectic nuclei observed at 12 and 24 hr were positive for the modified TUNEL stain (Fig. 2).

A few cells showing shrinkage and round-shape were detected in the PC12 cell culture at 6 hr after the addition of 5AzC, while the cells were shown to be intact up to 3 hr (Fig. 3a-d). Most cells showed such changes and were detached from the culture plate at 12 and 24 hr after 5AzC-treatment.

Fragmented DNA detected by the ELISA method slightly increased at 6 hr (1.13) and a prominent rise was observed at 12 and 24 hr (5.73 and 6.61, respectively) after 5AzC-treatment (Table 1). At 36 hr, the value decreased but remained higher compared with that before treatment.

Methylation state of genomic DNA after 5AzC -treatment

If the most CCGG sites of genomic DNA are demethylated, MspI digestion and HapII digestion would result in the same elecrophoretic pattern. However, the patterns were different up to 24 hr after 5AzC-injection (Fig. 4). The same results were obtained in the case of

Table 1. DNA fragmentation of PC12 cells after 5AzC-treatment measured by ELISA

TIME AFTER 5AzC (hr)	OD SAMPLE/OD PRE
0	1.00
6	1.13
12	5.73
24	6.61
36	2.28

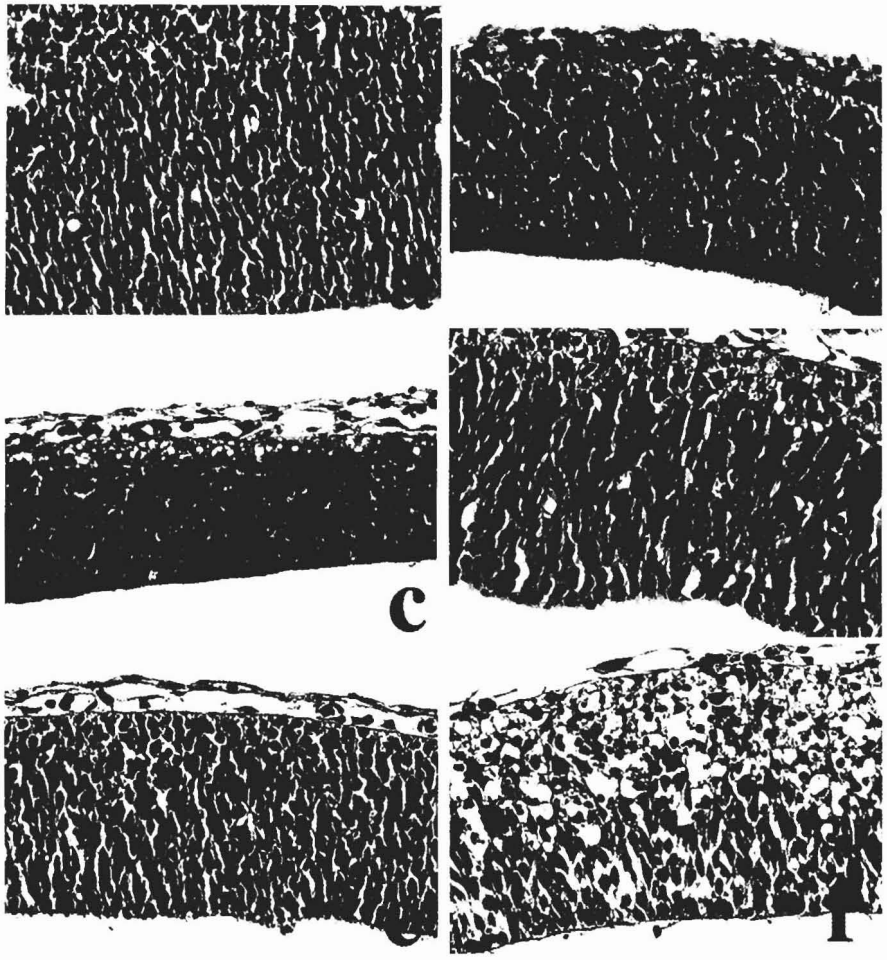

Fig. 1. The brain wall of the fetuses from the dams injected with 5AzC. At 0 hr **(a)**, 1 hr **(b)**, 3 hr **(c)**, 6 hr **(d)**, 12 hr **(e)** and 24 hr **(f)** after the injection. Pyknotic or karyorrhectic nuclei are first observed at 12 hr and their number increases at 24 hr. HE stain. x 250

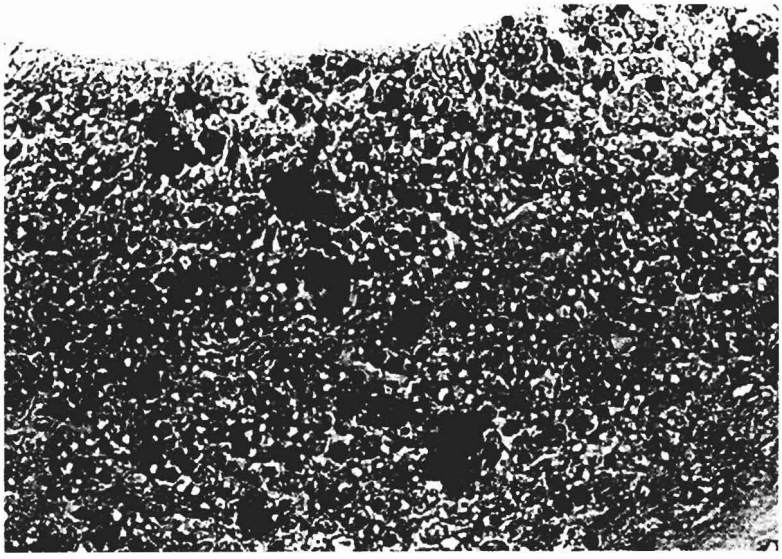

Fig. 2. A number of apoptotic cells are seen in the brain wall of a fetus from the dam injected with 5AzC. 24 hr after the injection. A modified TUNEL method (ApopTag). x 480

PC12 cells (Fig. 5). This indicated that the change of genomic DNA methylation state was under a detectable level in the fetal brain cells and PC12 cells treated with 5AzC.

Apoptotic effect of cytidine analogues and some other nucleosides

5AzC and 5AzdeC induced apoptosis in the brain and spinal cord of the fetuses at 24 hr after the injection to dams (Table 2), while the other chemicals tested (6AzC, 5Azcytosine, cytidine, 3deAzA, 3deAzU and 6AzU) failed to induce apoptosis. Figure 6 shows the brain histopathology of the fetuses at 24 hr after the treatment of the chemicals. Apoptotic lesions were observed in the migratory zone of the brain wall and in the mantle layer of the spinal cord of the fetuses treated with 5AzC (Fig. 6a) or 5AzdeC (Fig. 6b). No lesions were detected in the brain of fetuses treated with any other chemicals (Fig. 6c,d).

In the system of PC12 cells, only 5AzC induced morphological cell changes characteristic of apoptosis (Fig. 7). The results of ELISA also revealed an increase in DNA fragmentation in the cells treated with 5AzC (Table 3). 3deAzA induced a considerable level of DNA fragmentation without any morphological changes. However, other chemicals, including 5AzdeC which

Table 2. Induction of apoptosis in the fetal brain and spinal cord by cytidine analogues and other nucleosides.

CHEMICALS	APOPTOSIS/TOTAL*
5AzC	15/15
5AzdeC	14/14
6AzC	0/14
5Azcytosine	0/15
Cytidine	0/13
6AzU	0/17
3deAzA	0/12
3deAzU	0/16

*: number of fetuses with neuronal apoptosis/number of total fetuses.

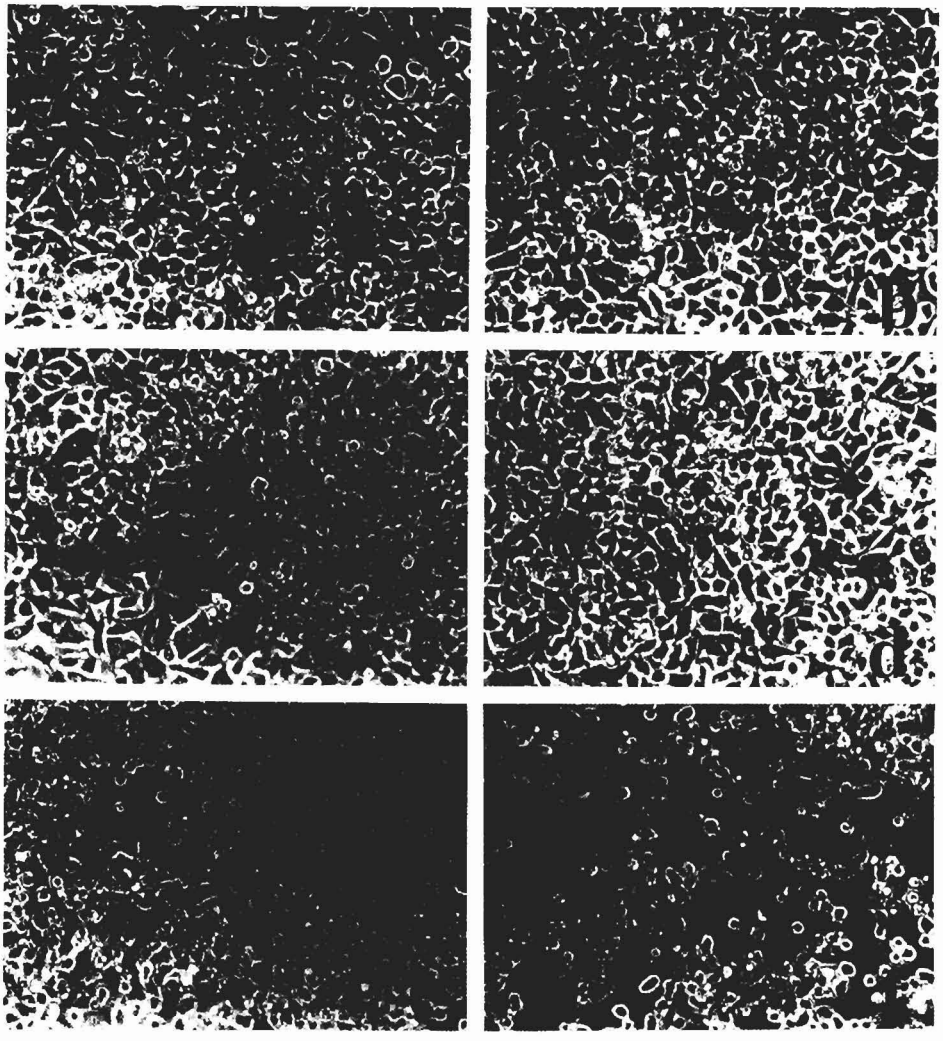

Fig. 3. PC12 cells treated with 5AzC. At 0 hr **(a)**, 1 hr **(b)**, 3 hr **(c)**, 6 hr **(d)**, 12 hr **(e)** and 24 hr **(f)**. Shrunk and round-shaped cells are seen in the culture at 6 hr or later. The changes are prominent at 12 hr and 24 hr. Phase-contrasted microscopy. x 180

induced apoptosis in the *in vivo* system, failed to provoke morphological and biochemical changes characteristic of apoptosis (Table 3).

Ability of 5AzC to induce apoptosis in C6 cell cultures

Three cytidine analogues including 5AzC, 5AzdeC and 6AzC failed to induce not only morphological changes characteristic of apoptosis but DNA fragmentation in C6 cells (Fig. 8 and Table 4). On the other hand, only 5AzC induced DNA fragmentation in PC12 cells (Fig. 8 and Table 4).

Discussion

In the present study, we used a commercially

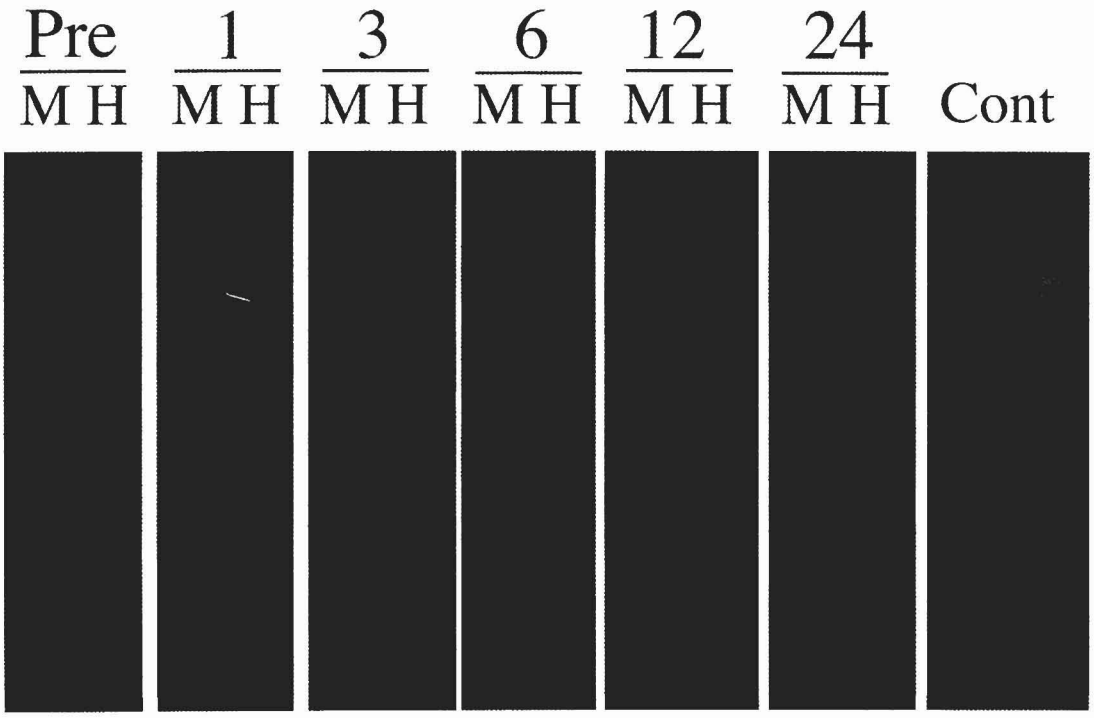

Fig. 4. Elecrophoretic pattern after Msp I (M) or Hap II (H) digestion. DNA samples were extracted from fetal brains. Fetuses were removed from the dams injected with 5AzC at the time indicated in the figure. The pattern of the two digests shows no change during the observation time. Pre: pretreatment. Cont: not digested.

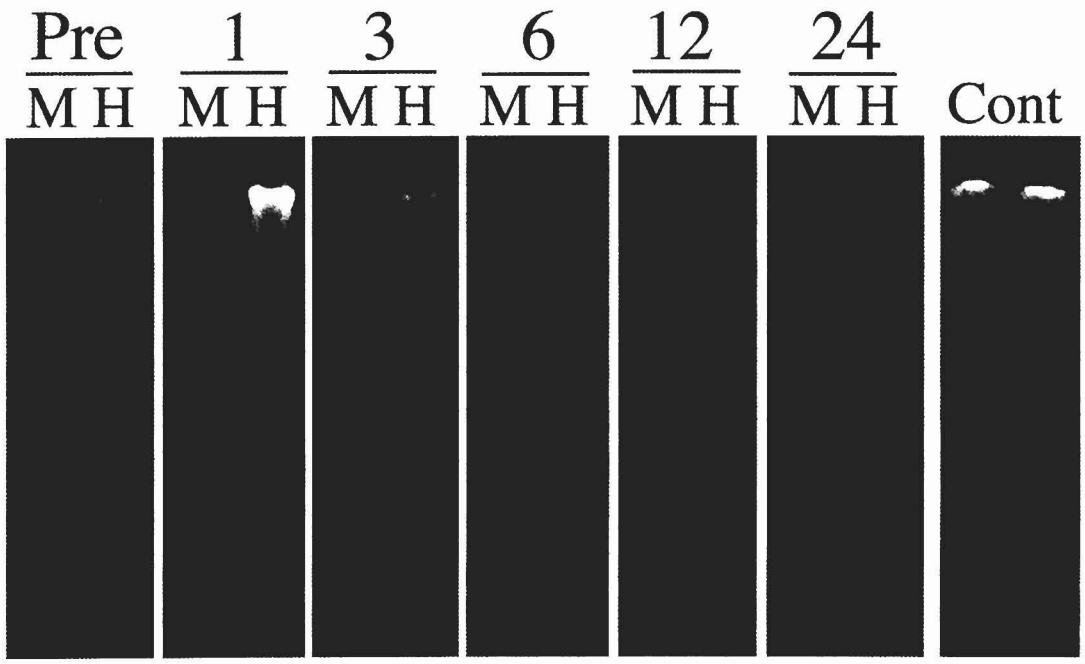

Fig. 5. Elecrophoretic pattern of Msp I (M) or Hap II (H) digestion. DNA samples were extracted from PC12 cells treated with 5AzC at the time indicated in the figure. The pattern of the two digestions shows no change during the observation time. Pre: pretreatment. Cont: not digested.

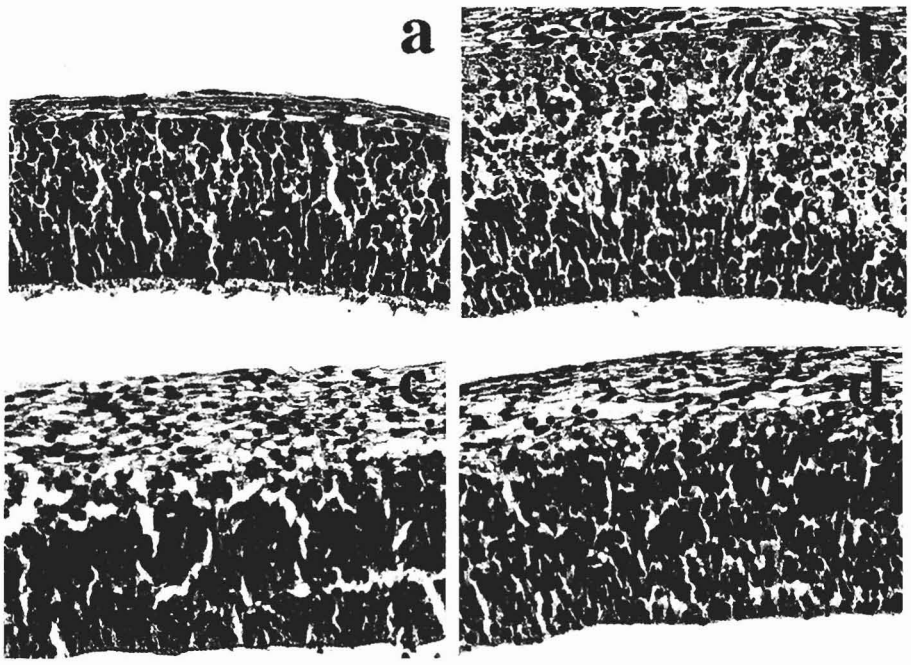

Fig. 6. Brain wall of the fetuses at 24 hr after the injection of 5AzC **(a)**, 5AzdeC **(b)**, 6AzC **(c)** or cytidine **(d)**. 5AzC and 5AzdeC induce apoptosis, while 6AzC and cytidine do not. HE stain. x 250

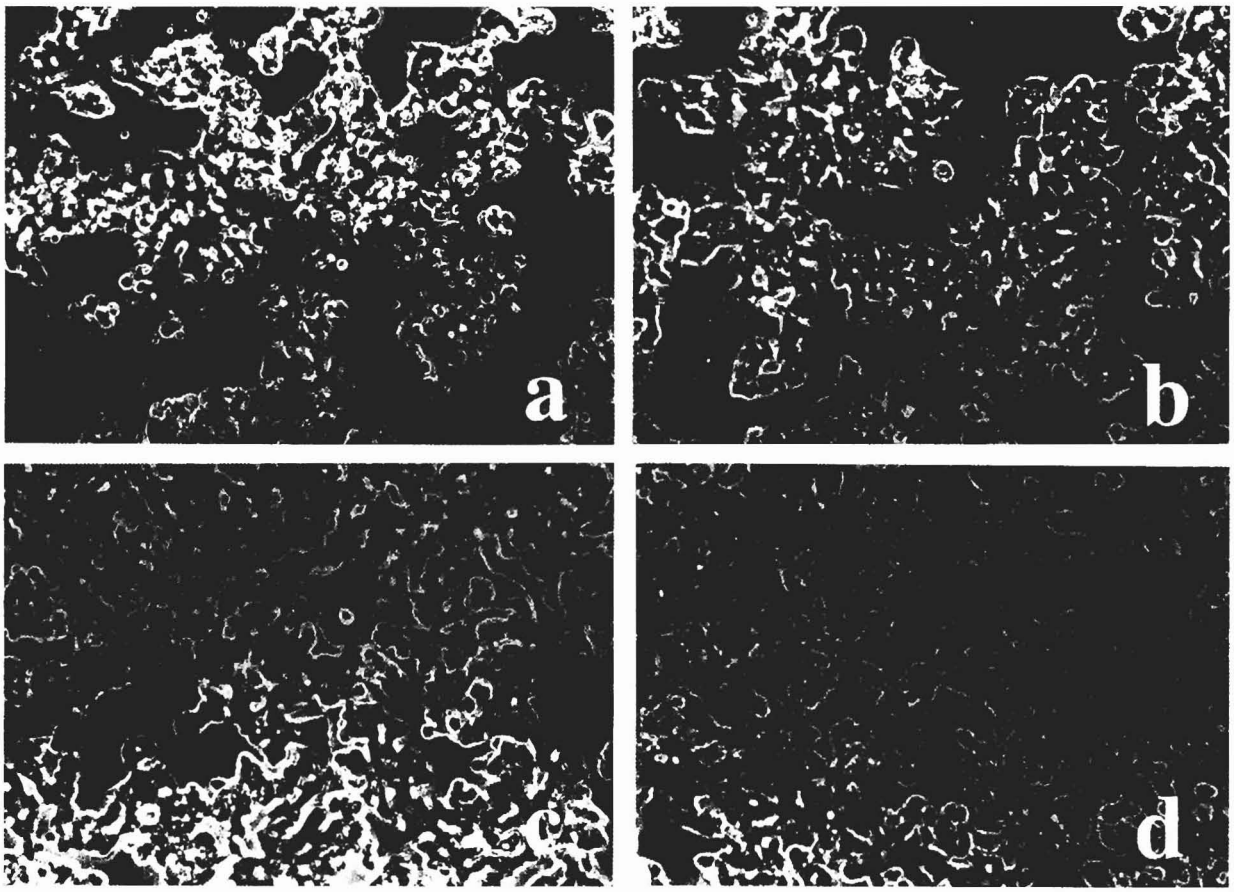

Fig. 7. PC12 cells at 24 hr after the treatment with 5AzC **(a)**, 5AzdeC **(b)**, 6AzC **(c)** or distilled water **(d)**. Cells treated with 5Az or 5AzdeC show shrinkage and round-shape. Phase-contrasted microscopy. x 300

available ELISA kit to detect apoptosis (DNA fragmentation). The results showed that DNA fragmentation started at 12 hr and peaked at 24 hr after the 5AzC-treatment. This was a very similar time course with the morphological appearance of apoptosis described in the previous (Hossain et al., 1995) and present studies. The ELISA method, therefore, is considered to be useful for evaluating the 5AzC-induced neuronal apoptosis.

Among 8 chemicals used in the present study, only 5AzC and 5AzdeC were able to induce apoptosis in the fetal brain. When DNA methylation occurs, cytosine base is methylated at the 5 position of pyrimidine ring. In the cells treated with these two analogues, cytosine bases are replaced by nitrogen-substituted cytosines (5-azacytosine), and the DNA that had taken the analogues would be unable to be methylated because the methyl residue could not bind to the 5 position. On the other hand, replacement at position 6 failed to induce apoptosis. In addition, cytosine itself was unable to induce apoptosis, probably because it cannot be taken

Table 3. Induction of DNA fragmentation in PC12 cells by cytidine analogues and other nucleosides.

CHEMICALS	OD SAMPLE/OD CONT.
None (Cont)	1.00
DW	0.78
5AzC	14.14
5AzdeC	1.46
6AzC	0.84
5Azcytosine	0.93
Cytidine	1.29
6AzU	1.09
3deAzA	6.63
3deAzU	1.77

Table 4. Induction of DNA fragmentation in PC12 and C6 cells.

CELLS	CHEMICALS	OD SAMPLE/OD CONT.
PC12	Cont.	1.00
	5AzC	8.95
	5AzdeC	0.79
C6	Cont.	1.00
	5AzC	-1.24
	5AzdeC	0.76
	6AzC	0.94

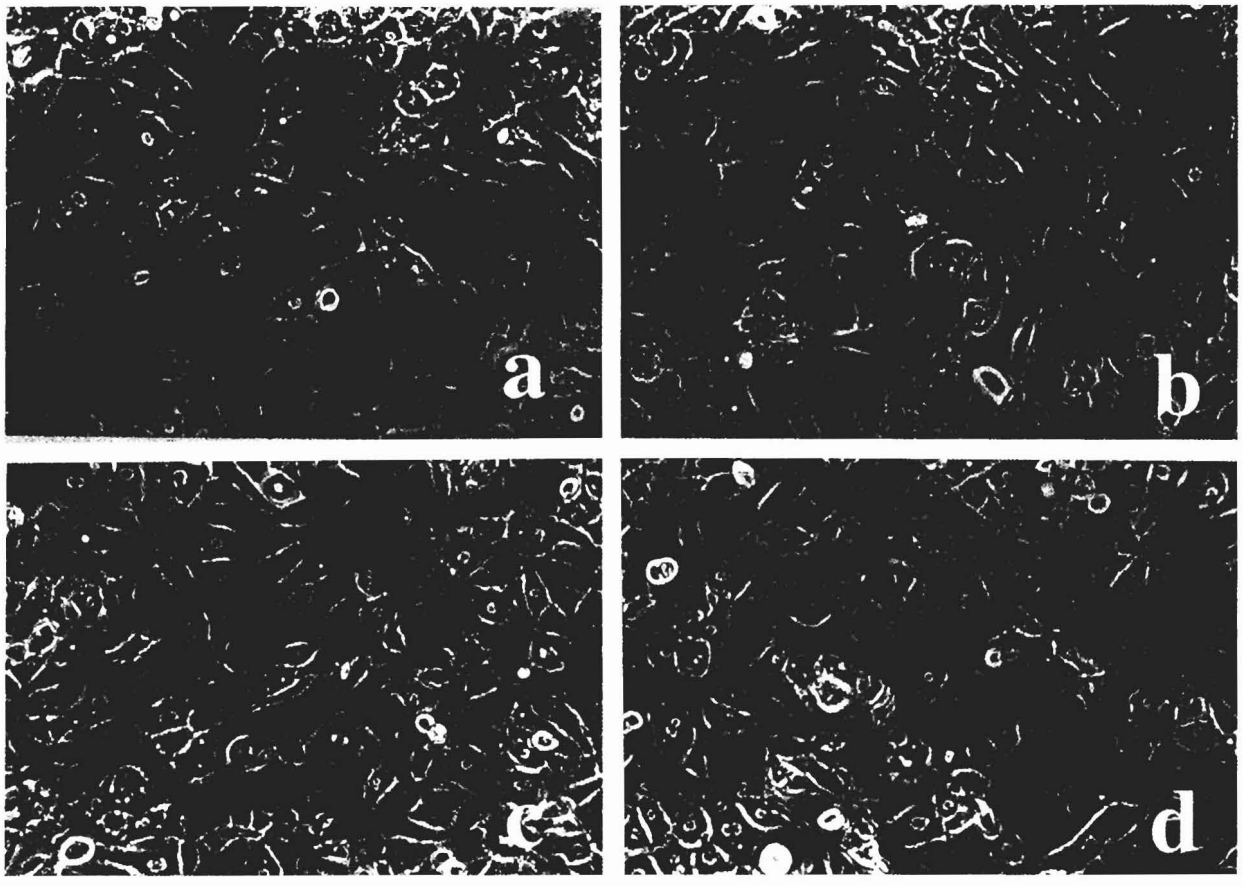

Fig. 8. C6 cells at 24 hr after the treatment with 5AzC **(a)**, 5AzdeC **(b)**, 6AzC **(c)** or distilled water **(d)**. No changes are observed after any treatment. Phase-contrasted microscopy. x 300

into DNA. Cytosine is not a nucleoside as it lacks sugar component. From these results, it seems that the replacement of carbon atom with nitrogen atom at the 5 position of the pyrimidine ring is required for demethylation of DNA and for subsequent apoptosis in mouse fetuses. Stopper et al. (1995) reported that 5AzC analogs having more chemically accessible 5 positions (5,6-dihydro-5-azacytidine and 6AzC) are unable to induce apoptosis.

On the contrary, 5AzdeC failed to induce apoptosis (DNA fragmentation) in the *in vitro* system using PC12 cells, while it induced apoptosis in the *in vivo* system. The probable reasons for the difference between *in vivo* and *in vitro* systems are as follows; i) the uptake of 5AzdeC into PC12 cell might be less as compared to that into fetal brain cells; ii) the control mechanism of DNA methylation might be different between fetal brain cells and PC12 cells; or iii) other mechanisms of apoptosis far from DNA methylation would be involved. However, there has been no evidence to support them until the present.

Therefore, we examined the methylation state of genomic DNA after 5AzC-treatment, but were unable to detect any changes in DNA methylation state in either the *in vivo* or *in vitro* experiments. The most plausible explanation for these results is that the DNA demethylation probably occurs only in the promotor region of a certain apoptosis-related gene(s) and the detection level of the method used may be less than the limit. We are now trying to identify possible genes which can be demethylated following the 5AzC-treatment by the technique of DNA subtraction using PC12 cells.

In the present study, 5AzC could induce apoptosis in PC12 cells, which are derived from rat pheochromocytoma and considered to be primitive neuronal cells without nerve growth factor (NGF)-treatment, but not in C6 cells derived from rat glioma. Our previous *in vivo* study (Hossain et al., 1995) showed that only undifferentiated brain cells in the migratory zone of the fetal brain wall were affected with apoptosis and that differentiated neurons and glial cells in the cortical layer were not. These results indicate that only the neuronal cells with primitive nature are the target of 5AzC. The details of the cell-specific action of 5AzC to induce apoptosis has to be clarified further in future.

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