

5-Azacytidine (5Az) induces apoptosis in PC12 cells: a model for 5Az-induced apoptosis in developing neuronal cells

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Summary. Our previous *in vivo* and *in vitro* studies showed that 5-azacytidine (5Az), a cytidine analog, induced apoptosis in developing neuronal cells in mice. To develop a system in which the precise molecular mechanism of 5Az-induced apoptosis in developing neuronal cells could be elucidated, we carried out the present study with PC12 cells. These cells are derived from a rat pheochromocytoma and extrude neurites in response to nerve growth factor (NGF). Light microscopy showed dose-dependent pyknotic and karyorrhectic changes in undifferentiated PC12 cells. Although they were less sensitive to 5Az, NGF-treated differentiated cells showed the same changes. Analysis by the TUNEL method (an *in situ* method for the detection of apoptosis) showed positive signals in the pyknotic and fragmented nuclei of these cells. Transmission electron microscopy revealed margination, segmentation, and condensation of nuclear chromatin, cell body shrinkage, and cytoplasmic vacuolization. Scanning electron microscopy demonstrated bleb formation on the cell surface. These pathomorphological changes are typical of apoptosis. 5Az seemed to affect cells that were in the proliferative stage; when the cells were terminally differentiated, their sensitivity to 5Az appeared to decline. PC12 cells could be used as a pathomorphological and biochemical model for studies of 5Az-induced neuronal cell apoptosis at the molecular and genetic level.

Key words: Apoptosis, 5-Azacytidine, Central nervous system (CNS), PC12 cells

Introduction

The cytidine analog, 5-azacytidine (5Az), has been shown to induce cell differentiation in various systems

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(Taylor and Jones, 1979; Cedar and Razin, 1990). Studies of the effects of 5Az on cell function and differentiation have demonstrated that the incorporation of 5Az into genomic DNA leads to its hypomethylation (Jones and Taylor, 1980; Creusot et al., 1982; Jones, 1985) and thus, possibly, allow inactive genes to become expressed (Iguchi-Arigo and Schaffner, 1989). This agent is, in addition, highly toxic in cultured cells (Flatau et al., 1984; Davidson et al., 1992) and in animals (Jaenisch et al., 1985; Richel et al., 1988). For this reason, 5Az is utilized as a potent anticancer agent for the treatment of myeloid leukemia and myelodysplastic syndromes (Pinto and Zaganel, 1993).

Pathomorphological investigations of the effects of 5Az on the developing central nervous system (CNS) showed significantly elevated apoptotic cell death (Hossain et al., 1995, 1996). Nonspecific translation of some apoptotic proteins by the activation of certain genes through the demethylation mechanism was speculated to be responsible for these effects. Recently, 5Az-induced developmental arrest has been reported in the early chick embryo, concomitant with the synthesis of new protein (Zagris and Podimatas, 1994), supporting this speculation. However, contrasting results regarding the demethylation mechanism have been reported. Using methyl transferase-deficient mice, Jüttermann et al. (1994) showed that the cytotoxicity of 5-aza-2'-deoxycytidine, another cytidine analog, was attributable primarily to the covalent trapping of DNA methyltransferase and the consequent direct destruction of DNA rather than to the secondary demethylation of genomic DNA and subsequent protein synthesis.

In the fetal development of the nervous system, physiological programmed cell death plays a critical role in constructing a normally functioning neural network (Kallen, 1965; Hankin et al., 1988; Ellis et al., 1991; Oppenheim, 1991). Pathological or 5Az-induced apoptosis in the developing CNS often causes fetal mortality (Hossain et al., 1995). The establishment and maintenance of pure primary brain cell cultures from particular embryonal stages of development are required

to assess the molecular mechanism of this type of chemically induced apoptosis. However, the maintenance of primary neuronal cultures *in vitro* and the use of mouse fetuses in pregnant dams as an *in vivo* system are technically difficult (Hossain et al., 1995, 1996).

To establish a pure *in vitro* model for 5Az-induced neuronal apoptosis, we therefore, conducted a pathomorphological study of the effects of 5Az in PC12 cells, a rat pheochromocytoma cell line which differentiates into sympathetic neuron-like cells following the addition of nerve growth factor (NGF) (Greene and Tischler, 1976).

Materials and methods

Chemicals and growth factors

5-Azacytidine (5Az, Sigma Chemical Co., St. Louis, MO) and nerve growth factor (NGF, Takara Shuzo Co. Ltd., Japan) were used in this study.

Cell culture

The rat pheochromocytoma cell line PC12 was maintained in Dulbecco's minimum essential medium (DMEM) containing 5% bovine fetal serum (BFS) and 5% horse serum and incubated in a humidified atmosphere of 5% CO₂ at 37 °C. The cells (1x10⁶/ml) were cultured on cover slips in Petri dishes (Sumilon, Tokyo, Japan). Non-neuronal PC12 cells were cultured for 1 week and 5Az was then added at different concentrations (0, 5, 50, 100, and 500 µg/ml). Neuronal PC12 cells were prepared by treating the cell with NGF (50 ng/ml) in DMEM containing FBS and horse serum for 4 days. After this time, neurites had spread out and the cells seemed to have stopped proliferating. 5Az was added at different concentrations (0, 5, 50, 100, and 500 µg/ml) 4 days after the addition of NGF. Thirty hours after the addition of 5Az, both neuronal and non-neuronal cells were fixed with Bouin's fluid for hematoxylin and eosin (HE) staining or with methanol for Giemsa staining, and examined with a light microscope.

In situ detection of apoptosis by modified TUNEL method

This method has been used to detect the *in situ* nucleosomal DNA fragmentation characteristic of apoptosis (Gavrieli et al., 1992). The principle of the method is as follows; at first the nuclear DNA is exposed by proteolytic treatment, carried out with proteinase K (Sigma Chemical Co., St. Louis, MO). Multiple fragmented DNA 3'-OH ends, which are specific for the nucleosomal fragmentation of DNA, are labeled with digoxigenin labeled-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT), and then reacted with peroxidase-conjugated anti-digoxigenin antibody. The apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. A commercially

available detection kit, Apog TagTM (Oncor, Gaithersburg, MD), was used.

Electron microscopy

For transmission electron microscopy (EM), the cells were washed gently with phosphate-buffered saline (PBS), pelleted by mild centrifugation, fixed with 2.5% glutaraldehyde in 0.1M PBS (pH 7.4), postfixed with 1% osmium tetroxide in the same buffer, dehydrated through a graded series of ethanol and propylene oxide, and embedded in epoxy resin (Epon 812; Oken, Tokyo, Japan). Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined under a Jeol 1200 EX transmission electron microscope. For scanning electron microscopy (SEM), the cells were cultured on collagen-coated coverslips (Sumilon, Tokyo, Japan) in Petri-dishes, fixed with 1% glutaraldehyde in 0.1M PBS (pH 7.4), postfixed with 1% osmium tetroxide in the same buffer, dehydrated through an ascending series of ethanol and isoamyl acetate, and dried by a CO₂ critical point method. The specimens were ion-coated with a thin layer of platinum and palladium and examined under a Hitachi S-4000 scanning electron microscope.

Quantitative analysis

On the Apog TagTM-stained plates the cells, both positive and negative were counted in 10 randomly selected fields under a light-microscope at x200 magnification. Positive cells were expressed as a percentage of the total number of cells counted per field.

Results

A. Undifferentiated PC12 cells (without NGF)

Light microscopy

The control undifferentiated PC12 cells (without 5Az treatment) were spherical to round in shape with round nuclei and prominent nucleoli; they frequently showed mitosis (Fig. 1a). Few of the cells treated with 5 µg/ml of 5Az developed pyknosis. In contrast, cells treated with 5Az, at doses of 50 and 100 µg/ml, developed significant number of pyknotic and karyorrhectic changes (Fig. 1b). Light microscopic changes of 5Az-treated undifferentiated PC12 cells were considered to be apoptotic cell death morphologically.

In situ detection of apoptosis

Undifferentiated cells treated with 5Az showed Apog Tag-positive signals, indicated by the brownish coloration of seemingly intact as well as fragmented nuclei (Fig. 2). Fig. 9 shows the percentage of Apog Tag-positive cells in 5Az-treated undifferentiated PC12 cell culture. A very low incidence (3.2±0.6%) of

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apoptotic cells was found in the control group (0 mg/ml). A low dose of 5Az (5 μ g/ml) induced apoptosis in $4\pm 1.2\%$ of the cells. 5Az at 50 mg/ml caused apoptosis in $24\pm 2.4\%$ of the cells while 5Az at 100 μ g/ml caused apoptosis in $42.8\pm 2.5\%$ of the cells.

TEM

The control undifferentiated PC12 cells (without 5Az) showed round nuclei with abundant electron-lucent chromatin and prominent nucleoli; they also exhibited abundant cytoplasmic organella such as mitochondria and endoplasmic reticulum (Fig. 3a). In 5Az-treated cultures, margination, segmentation, and condensation of nuclear chromatin, shrinkage of the cell, and numerous enlarged cytoplasmic vacuoles typical of apoptosis were observed (Fig. 3b) mainly in the affected cells.

SEM

Undifferentiated PC12 cells without 5Az or those

treated with 5 μ g/ml of 5Az showed a normal appearance, characterized by a spherical or polygonal shape; the cells tended to grow in small clumps with an almost smooth cell surface (Fig. 4a), while many cells treated with 100 μ g/ml of 5Az showed bleb formation on the cell surface or apoptotic bodies (Fig. 4b).

B. Differentiated PC 12 cells (with NGF)

Light microscopy

After the addition of NGF, observation under a phase contrast microscope revealed that the morphology of undifferentiated PC12 cells had changed from spherical or round to pyramidal and the cells had differentiated into neurons that extruded cellular processes (Fig. 5). The control differentiated PC12 cells (without 5Az) showed a very low proportion of pyknotic change and maintained normal growth as pyramidal cells with the extrusion of neuronal processes (Fig. 6a). Cells treated with 5 μ g/ml of 5Az were morphologically

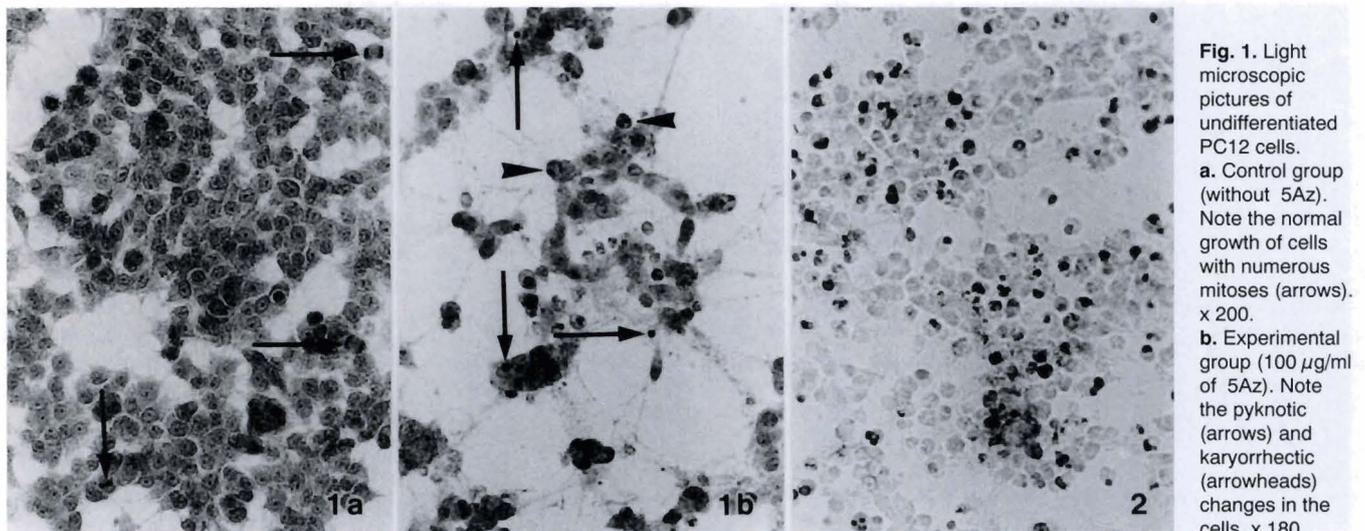


Fig. 1. Light microscopic pictures of undifferentiated PC12 cells. **a.** Control group (without 5Az). Note the normal growth of cells with numerous mitoses (arrows). x 200. **b.** Experimental group (100 μ g/ml of 5Az). Note the pyknotic (arrows) and karyorrhectic (arrowheads) changes in the cells. x 180

Fig. 2. *In situ* detection of apoptosis by a modified TUNEL method in undifferentiated PC12 cells. Experimental group (100 μ g/ml of 5Az). Note the positive signals of apoptosis in seemingly intact, pyknotic and karyorrhectic nuclei of numerous cells. x 180

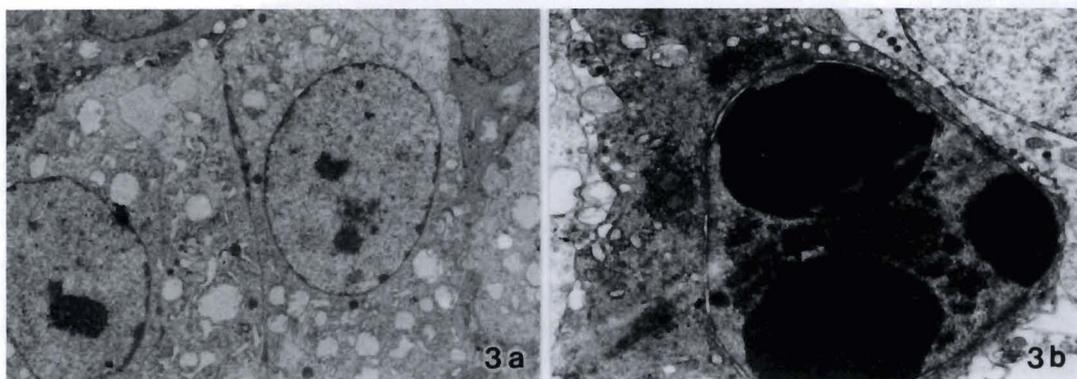


Fig. 3. Transmission electron microscopic pictures of undifferentiated PC12 cells. **a.** Control group (without 5Az). No changes were observed. x 4,300. **b.** Experimental group (100 μ g/ml of 5Az). Note the condensation and segmentation of the nucleus. x 12,000

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similar to the control differentiated PC12 cells. Treatment of the differentiated PC12 cells with 50 µg/ml of 5Az induced fewer pyknotic and karyorrhectic changes than those seen in undifferentiated PC12 cells treated with 5Az at the same dose. Differentiated PC12 cells treated with 100 and 500 µg/ml of 5Az developed

considerable pyknotic and karyorrhectic changes (Fig. 6b).

In situ detection of apoptosis

Differentiated PC12 cells treated with 5Az showed

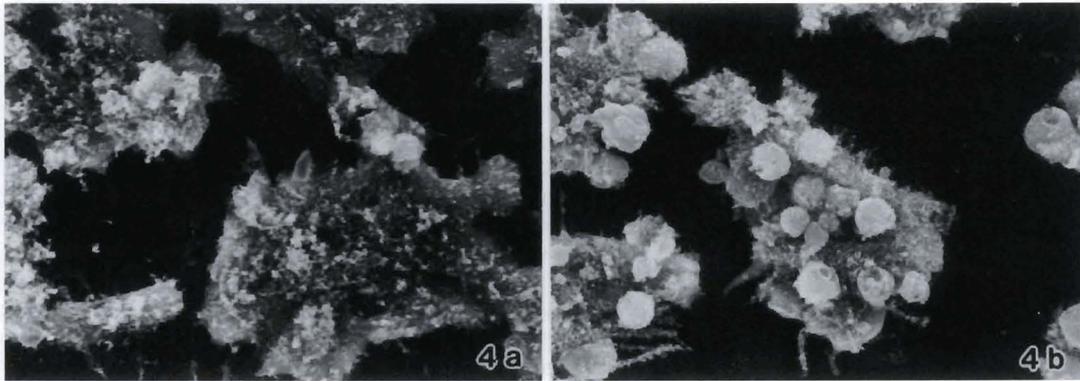


Fig. 4. Scanning electron microscopic pictures of undifferentiated PC12 cells. **a.** Control group (without 5Az). Note the normal growth and lack of prominent changes on the cell surface. x 1,150. **b.** Experimental group (100 µg/ml of 5Az). Note the bleb formation (apoptotic bodies) on the cell surface. x 1,650

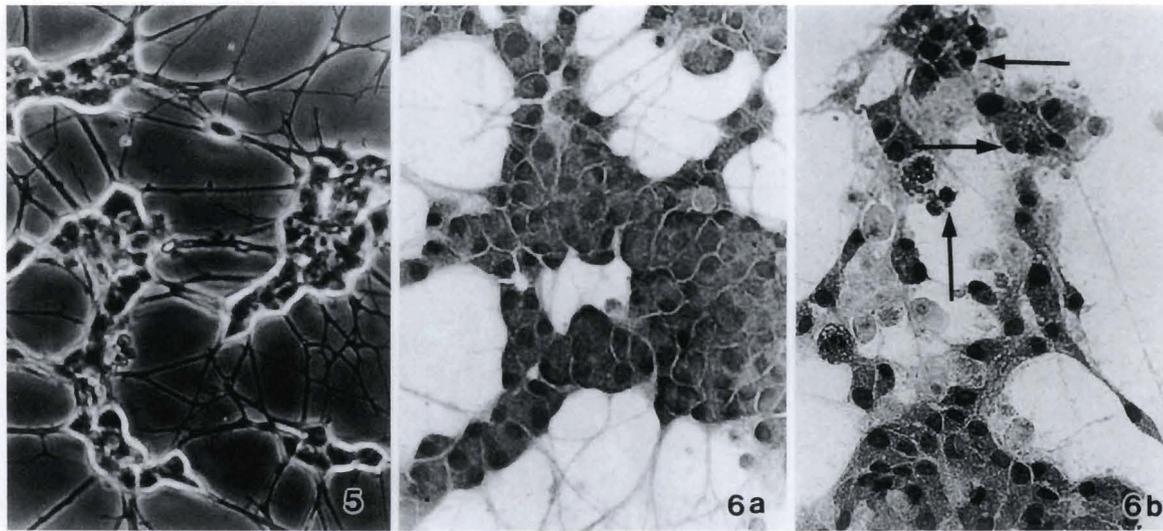


Fig. 5. Phase contrast microscopic picture of NGF-treated differentiated PC12 cells. Many neurites can be seen connected with each other. x 200

Fig. 6. Light microscopic pictures of differentiated PC12 cells. **a.** Control group (without 5Az). Note the normal growth of cells

with the extrusion of neurites connected to each other. x 240. **b.** Experimental group (500 µg/ml of 5Az). Note the few pyknotic nuclei (arrows) of round-shaped cells. x 240

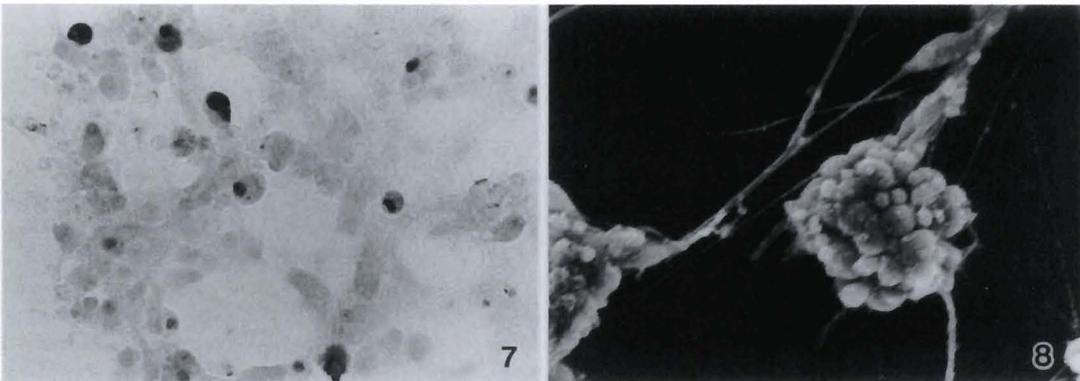


Fig. 7. *In situ* detection of apoptosis by a modified TUNEL method in differentiated PC12 cells. Experimental group (500 µg/ml of 5Az). Note the positive signals of apoptosis indicated in the pyknotic and karyorrhectic nuclei. x 240

Fig. 8. Scanning electron microscopic picture of differentiated PC12 cells. Experimental group (500 µg/ml of 5Az). Note the

apoptotic bodies and degenerated processes. x 720

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positive apoptotic signals mainly in round-shaped and scarcely in pyramidal cells (Fig. 7) but not in cells with developed neurites. Fig. 10 shows the percentage of Apop Tag-positive cells in 5Az-treated differentiated PC12 cells. The control group (5Az, 0 $\mu\text{g/ml}$) exhibited apoptosis at the rate of $3.4 \pm 0.9\%$, the physiological level. 5Az, at 5, 50, and 100 $\mu\text{g/ml}$ induced apoptosis in $4.7 \pm 0.9\%$, $5.9 \pm 0.6\%$, and $8.5 \pm 0.8\%$ of cells, respectively, whereas 500 $\mu\text{g/ml}$ of 5Az induced apoptosis in $13 \pm 2.3\%$ of the cells.

SEM

Some PC12 cells treated with 500 $\mu\text{g/ml}$ of 5Az showed bleb formation on the cell surfaces, one of the characteristics of so-called apoptotic bodies (Fig. 8). The control differentiated PC12 cells (without 5Az) were star- or pyramid-shaped and connected to each other with well developed neurites.

Discussion

The main purpose of this experiment with undifferentiated and differentiated PC12 cells was to establish a suitable system by which the details of the molecular mechanism of 5Az-induced neuronal apoptosis could be elucidated. Light microscopic investigation revealed pyknotic and karyorrhectic changes, morphologically characteristic of cell death, mainly in less differentiated cells. *In situ* detection of apoptosis showed positive signals in seemingly intact nuclei as well as in the pyknotic and karyorrhectic nuclei of less differentiated cells. TEM showed margination, segmentation, and condensation of nuclear chromatin and vacuolization of the cytoplasm, the typical morphological characteristics of apoptosis. In addition, SEM showed typical apoptotic bodies. These findings

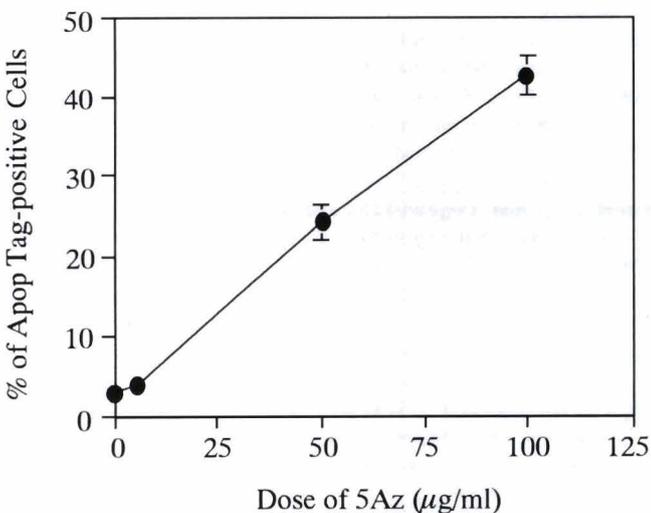


Fig. 9. 5Az-treated undifferentiated PC12 cells. Percentage of Apop Tag-positive cells.

are very much typical for apoptosis, indicating that 5Az also induces apoptosis in a neuronal cell line.

The present experiment revealed that the effect of 5Az on PC12 cells was dose-dependent and that the differentiated neuronal cells were much more resistant than the undifferentiated cells and primary brain culture cells examined in our previous study (Hossain et al., 1996). These results suggest that sensitivity to 5Az is reduced with differentiation, and that this agent may induce apoptotic cell death in cells at cell division (Takeuchi and Takeuchi, 1978) but not in the resting phase of the cell cycle. The present study also showed that the undifferentiated PC12 cells (without NF treatment) were fully replicating (indicated by morphology) and were more sensitive to 5Az than the NGF-treated PC12 cells (differentiated). In our previous study (Hossain et al., 1995), we reported that differentiating CNS cells were highly sensitive to 5Az at the fully dividing developmental stage. The response of PC12 cells without NGF-treatment to 5Az and the stage-specific response of the developing CNS cells appears to be similar.

Differentiated PC12 neuronal cells die as a result of apoptosis when deprived of NGF and this death can be prevented by the inhibition of mRNA or protein synthesis (Mesner et al., 1992). Our biochemical study (Hossain et al., 1997) has indicated that 5Az-induced apoptosis in differentiated PC12 cells (NGF-treated) was also inhibited by a protein synthesis inhibitor, suggesting the involvement of new protein synthesis in this type of chemically induced apoptosis. Evidence of such protein synthesis has also been observed in 5Az-treated early chick embryos that showed developmental arrest (Zagris

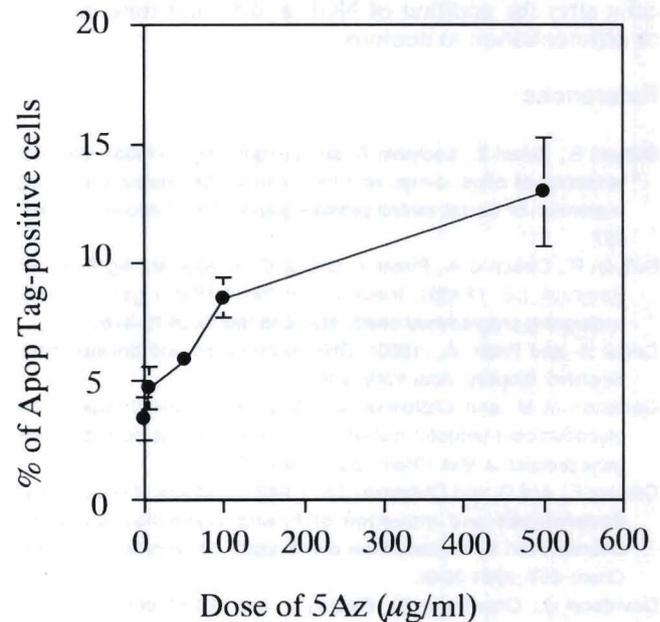


Fig. 10. 5Az-treated differentiated PC12 cells. Percentage of Apop Tag-positive cells.

and Podimatas, 1994). In various examples of non-neuronal programmed cell death (PCD), the expression of functional proteins such as nucleases (Compton and Cidlowski, 1987), polyubiquitin (Schwartz et al., 1990), hsp70, c-Fos, c-Myc (Buttayan et al., 1988), and TRPM-2 (Buttayan et al., 1989) has been detected in association with cell death. The expression of c-Jun in NGF-deprived sympathetic neurons has also been shown by some investigators (Estus et al., 1994; Schlingensiepen et al., 1994; Ham et al., 1995). In 5Az-induced apoptosis in PC12 cells, the identification of such kind of proteins and their precise functions is necessary for understanding the mechanism of this chemically induced apoptosis in neurons. A recent study has reported that the cell death induced by another cytidine analog, 5-aza-2'-deoxycytidine, was due to the direct toxicity of this agent acting through covalent methyltransferase trapping; the cell death did not require new protein synthesis (Jüttermann et al., 1994). The mechanism of 5Az-induced neuronal apoptosis is thus still controversial.

To investigate the molecular mechanism through which 5Az induces apoptosis in developing neuronal cells, we require the establishment of an appropriate neuronal cell line system consisting of a pure cell population that is easy to manipulate. PC12 cells that differentiate into neurons following the addition of NGF should serve the desired purpose for elucidating PCD in neurons. Native PC12 cells (without NGF treatment) which are less differentiated, and are fully dividing could be used as a suitable model for detecting the precise molecular mechanism of 5Az-induced apoptosis in the developing central nervous system. Further detailed studies are required to clarify the effects of 5Az on PC12 cells after the addition of NGF at different time courses of differentiation to neurons.

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