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1-Amino-4-phenyl-1,2,3,6-tetrahydropyridine and 1-Amino-4-phenylpyridinium Salt, the 1-Amino Analogues of Neurotoxins, MPTP and MPP+, Induce Apoptosis in PC12 Cells: Detection of Apoptotic Cells by Comet Assay and Flow Cytometric Analysis

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Abstract. *1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to induce parkinsonism in humans when it is oxidized to the 1-methyl-4-phenylpyridinium salt (MPP+). We previously reported the syntheses of 1-amino-4-phenyl-1,2,3,6-tetrahydropyridine (APTP) and 1-amino-4-phenyl-pyridinium salt (APP+), the 1-amino analogues of the dopaminergic neurotoxins, MPTP and MPP+, respectively, and demonstrated that both APTP and APP+ are cytotoxic to PC12 cells. In this study, we found that both APTP and APP+ induce apoptotic cell death in PC12 cells. Apoptosis was determined by the Comet assay and flow cytometric analysis. Prior to using the Comet assay for detection of apoptotic PC12 cells, Comet images of apoptotic and necrotic cells were first distinguished by using several standards. Comet images were classified into four groups (A to D) according to their shapes. Class D consisted of the apoptotic cells and was easily distinguished. We also demonstrated that apoptotic and necrotic PC12 cells can be easily differentiated and quantified using the convenient Comet assay.*

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to induce parkinsonism in humans when it is oxidized to the 1-methyl-4-phenylpyridinium salt (MPP^+) (1). (Figure 1). We previously synthesized 1-amino-4-phenyl-1,2,3,6-tetrahydropyridine (APTP) and 1-amino-4-phenylpyridinium salt (APP^+), the 1-amino analogues of the dopaminergic neurotoxins, MPTP and MPP^+, respectively, and reported that both APTP and APP^+ were cytotoxic to PC12 cells, while only MPP^+ was cytotoxic in the methyl analogue (2). These differences were supported by the fact that APTP is oxidized to the active form, APP^+, by MAO-A in PC12 cells, while MPTP can be oxidized to the active form MPP^+ by MAO-B, which does not exist in these cells (3). It is reported that the cytotoxic effect of MPP^+ is caused by the induction of apoptosis (4), however, no studies have been done on APTP and APP^+.

We conducted this investigation to determine whether apoptosis is involved in the cell death induced by APTP and APP^+, as it is with MPP^+. For detection of apoptosis, both the Comet assay and flow cytometric analysis were employed. The Comet assay is a convenient and useful method for detecting DNA degradation and apoptosis (5-8), however, only a few reports have been published describing DNA damage in PC12 cells (9, 10) and none describing the detection of apoptosis. In this study, we first determined the Comet image of apoptotic cells in order to establish the method. Using this system together with flow cytometric analysis, we confirmed that both APTP and APP^+ induce apoptosis in PC12 cells, as does MPP^+.

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Materials and Methods

Materials. Propidium iodide, 4',6-diamidino-2-phenylindole (DAPI), trypsin and RNase A were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA), agarose gel was from Nacalai Tesque, Inc. (Kyoto, Japan), proteinase K, Tris, EDTA and sodium laurylsarcosinate were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), ethidium bromide was from Aldrich Chemical Co., Inc. (Tokyo, Japan), and the Annexin V-FITC kit was from Trevigen, Inc. (Gaithersburg, MD, USA). The phosphate-buffered saline (PBS) used was Dulbecco's PBS. Flow cytometric analysis was carried out with a FACSort (Becton Dickinson, USA).

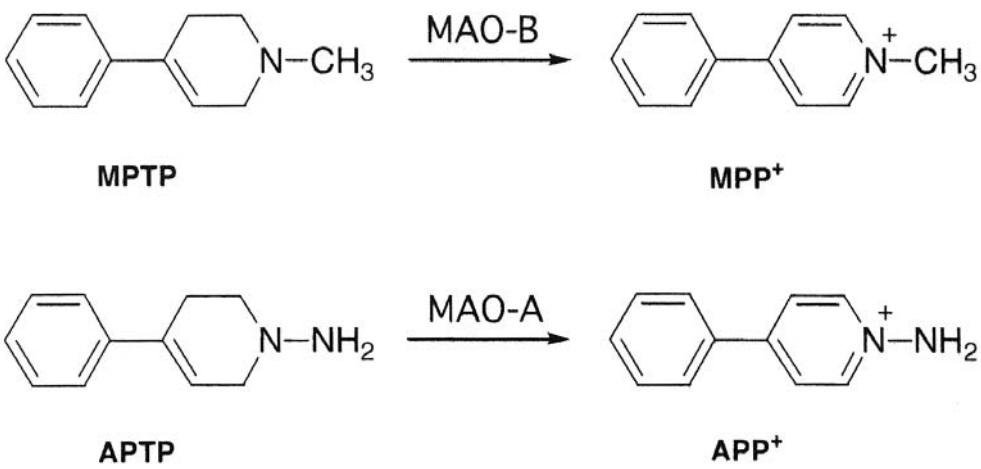

Figure 1. *Structures of MPTP, MPP+, APTP and APP+.*

Cell culture. Rat phenochromocytoma PC12 cells were cultured in Dulbecco's modified Eagle medium (Nissui PharmaceuticaI Co., Ltd., Tokyo, Japan) supplemented with 10% horse serum (Nacalai Tesque Inc., Kyoto, Japan), 5% fetal bovine serum (Summit Biotechnology Co., Ltd., Fort Collins, CO, USA) and 0.1 mg/ml kanamycin (Meiji Seika Co., Ltd., Tokyo, Japan) under a humidified atmosphere of 5% CO_2 in air at 37˚C.

Flow cytometric analysis of cells in the sub-G1 region. We used a previously reported method with a slight modification (11). Cells (2 x 10^6) were seeded in a 50-mm plastic dish containing 5 ml medium and pre-cultured for 24 h. The test compound (50 μl) dissolved in DMSO was added and the cells were incubated for a specified time. The medium was then removed and the cells were washed with 2 ml of 0.02% EDTA in PBS. After the cells had been detached with 1 ml of 0.125% trypsin solution (0.02% EDTA in PBS), 5 ml of medium was added and the cells were collected by centrifugation. They were then washed with 1 ml of PBS, resuspended in 1 ml of cold 70% ethanol and refrigerated for 30 min. Cells were collected by centrifugation and washed with 1 ml of PBS. The cell pellet was treated with 50 μl of RNase A solution (1 mg/ml PBS) and 500 μl of disodium hydrogenphosphate-citric acid buffer (prepared by mixing 192 ml of 0.2 M Na_2HPO_4 and 8 ml of 0.1 M citric acid), and the mixture was incubated at 37˚C for 30 min. Then, the cells were stained for 30 min at room temperature in the dark, after addition of a further 500 μl of disodium hydrogenphosphate-citric acid buffer and 50 μl of propidium iodide solution (1mg/ml H_2O). This cell suspension was passed through a nylon mesh and subjected to flow cytometric analysis of the sub-G1 region.

Flow cytometric analysis of cells in early apoptosis. An Annexin V-FITC kit was used according to the manufacturer's instructions. After the cells were treated with the test compound for 30 h, they were collected as described above. To the cell pellet (1 x 10^6 cells) was added 100 μl of Annexin V incubation reagent (which contains Annexin V conjugate and propidium iodide, prepared according to the manufacturer's instructions), and the cells were incubated for 15 min at room temperature in the dark. After a binding buffer (400 μl) had been added, the cell suspension was passed through a nylon mesh and subjected to flow cytometric analysis.

Comet assay. Our previously reported method was used (12-14). Cells were collected by centrifugation, washed with PBS and resuspended in 500 μl of PBS. Twenty μl of the cell suspension was then mixed with 140 μl of 1% low melting temperature agarose gel. A hundred μl aliquot of this mixture was rapidly pipetted onto frosted microscope slides and allowed to gel at 4˚C for 10 min. The slides were then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, l0 mM Tris-HCl buffer (pH 10), 1% sodium sarcosinate with 1% Triton X-100 and l0% DMSO) at 4˚C for l h, then placed in a high pH electrophoresis buffer (l mM EDTA, 300 mM NaOH) at 4˚C for 20 min before electrophoresis. Electrophoresis was conducted at 4˚C for 20 min at 25 V and 300 mA. The slides were then immersed twice in 0.4 M Tris-HCl buffer (pH 7.5) for 5 min each time, stained with DAPI (l μg/ml) and covered with coverslips. Comet images were observed at more than 200x magnification with a fluorescence microscope (BX60, Olympus Co., Tokyo, Japan) attached to a video camera (CCD X-2, Shimadzu Rika Instruments, Tokyo, Japan) connected to a display screen. Fifty images were randomly selected from each sample and their Comet lengths were measured on the screen and classified as A to D. The extent of DNA strand breakage was evaluated by comparing the population of class A to D to that of the control cells.

DNA gel electrophoresis. DNA fragmentation was monitored by agarose gel electrophoresis. A previously reported method was used with a slight modification (15). Cells (1 x 10^6) were washed with cold PBS, centrifuged at 4˚C and resuspended in 20 μl of 50 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 0.5% (w/v) sodium laurylsarcosinate and 0.5 mg/ml proteinase K. After 1-h incubation at 50˚C, 10 μl of 0.5 mg/ml RNase A was added. Incubation at 50˚C was continued for an additional hour followed by heating to 70˚C. The cell suspension was then mixed with 10 μl of 10 mM EDTA (pH 8.0) containing 1% (w/v) low melting temperature agarose,

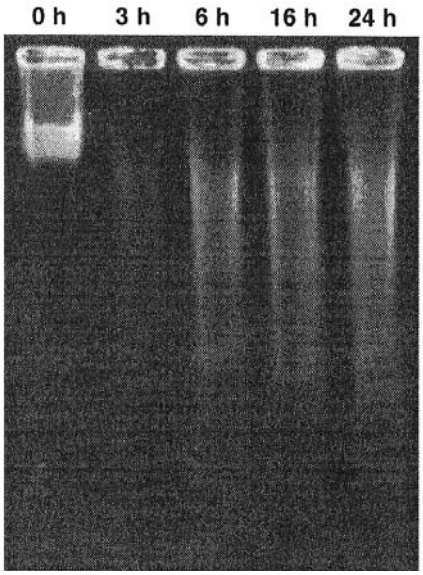

Figure 2. *Gel electrophoresis of PC12 cells starved for 3, 6, 16 and 24 h.*

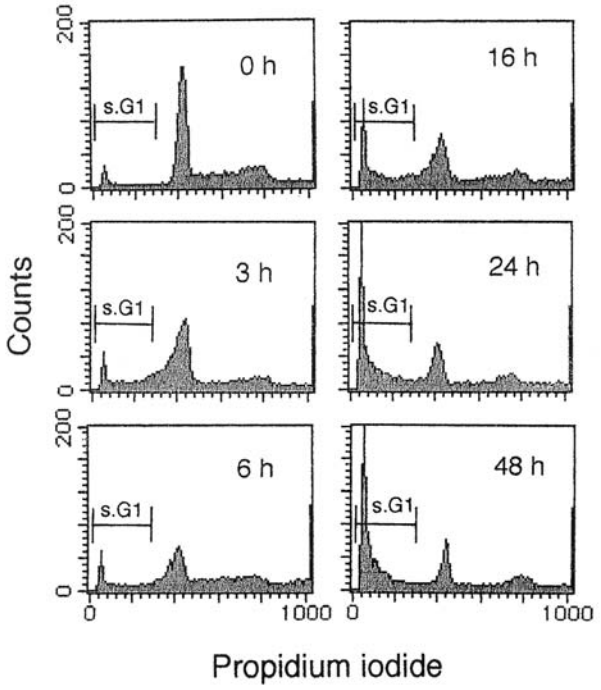

Figure 3. *Flow cytometry of PC12 cells starved for 3, 6, 16, 24 and 48 h. Detection of cells in the sub-G1 region. s.G1 shown in figure denotes the sub-G1 region.*

0.25% (w/v) bromophenol blue and 40% (w/v) sucrose, and loaded into wells prepared on a 1.2% (w/v) high melting temperature agarose gel plate. Electrophoresis was carried out at a voltage of 100 V in 2 mM EDTA–89 mM Tris-borate (pH 8.0) until the marker dye had migrated a distance of 3 ~ 4 cm. The agarose gel was then stained for 10 min with 0.1 μg/ml ethidium bromide.

Results and Discussion

The Comet assay (5-8), a microelectrophoresis technique, is a useful method for detecting DNA fragmentation in cells and widely used for the detection of DNA damage caused by mutagens/carcinogens. Our decision to use this method to analyze apoptotic PC12 cells was based on the fact that it proved satisfactory in our previous study (12-14). However, no report has been published on the detection of apoptosis in PC12 cells using the Comet assay. In this assay, Comet-like images with a head and tail can be observed, while the extent of DNA migration depends on the degree of DNA fragmentation. The DNA of apoptotic cells is fragmented by endonuclease, however, it was not clear which Comet image corresponded to the apoptotic cells. In order to ascertain this, apoptotic PC12 cells were prepared by a method in which the cells were cultured in a serum-deprived medium (16). To verify that apoptosis had been initiated, starved cells were analyzed by both agarose gel electrophoresis and flow cytometry and the induction of apoptosis was confirmed by the appearance of the ladder pattern (Figure 2) and the sub-G1 peak (Figure 3), respectively, which are characteristic of apoptotic cells. Starved cells were then subjected to the Comet assay. In order to compare these images with the typical Comet images induced by non-apoptotic cell death, cells treated with *N*-methyl-*N*-nitrosourea (MNU) were also subjected to the Comet assay (8). The Comet images observed with PC12 cells after starvation and treatment with MNU under several types of conditions were classified into four groups (A to D) as shown in Figure 4. Comet length was measured as the full length of the Comet, including the nucleus (the head portion) and tail, after electrophoresis. For comparison, the size of the Comet was expressed as the ratio of the full Comet length relative to the diameter of a normal nucleus. Figure 4-A shows the Comet image of control cells in which no, or a very small, tail can be observed, the size of the Comet being less than 1.2. Figure 4-B shows the appearance of a distinct tail and reduced nucleus, the size of the Comet being from 1.2 to 2.5. Figure 4-C shows a Comet with a big tail and a size of more than 2.5. The Comet images shown in Figures 4-B and 4-C were particularly characteristic of PC12 cells treated with MNU, as described later. Figure 4-D shows a highly diffused elliptical Comet tail and regression of the nucleus that may have been caused by extensive DNA degradation. This is a typical Comet image obtained with starved cells, and the cells in class D were determined to be

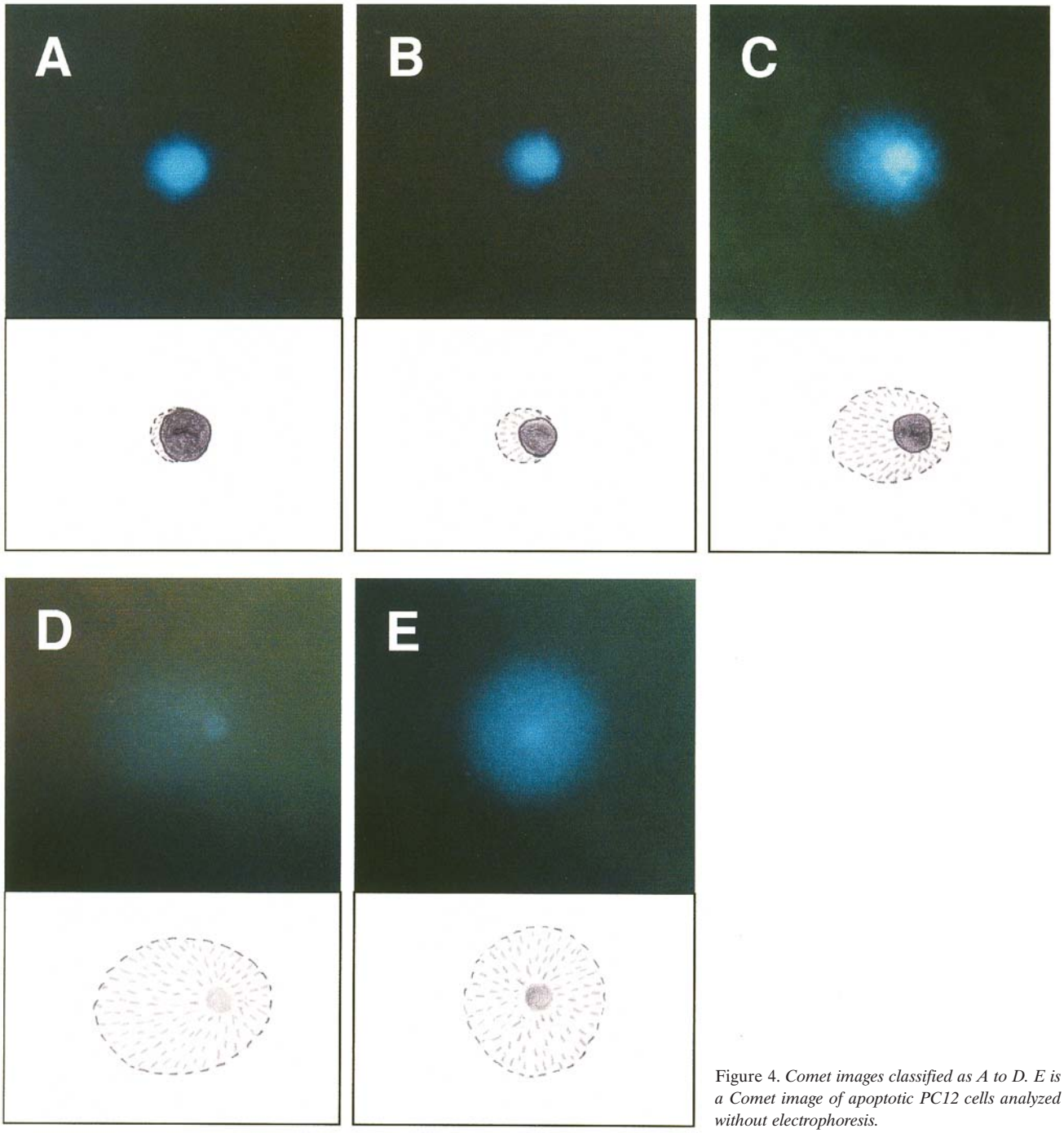

Figure 4. *Comet images classified as A to D. E is a Comet image of apoptotic PC12 cells analyzed without electrophoresis.*

apoptotic. Although the brightness of the Comet images of class D was weaker than that of the images of Figures 4-A to 4-C because of the high diffusion of the DNA fragments, these images are easily distinguishable from the Comet images of other cells with higher magnification (more than 200x). Furthermore, when the Comet assay was carried out without electrophoresis, a more typical image of apoptotic cells was observed, as shown in Figure 4-E, in which small DNA fragments are scattered around a small nucleus. This type of image was similar to that observed in the diffusion assay used by Singh (17) for the estimation of apoptotic cells.

Using this classification system, the population of cells was placed into class A to D after starvation and MNU treatment. Figure 5 shows the results of cells starved for 0 to 24 h. The

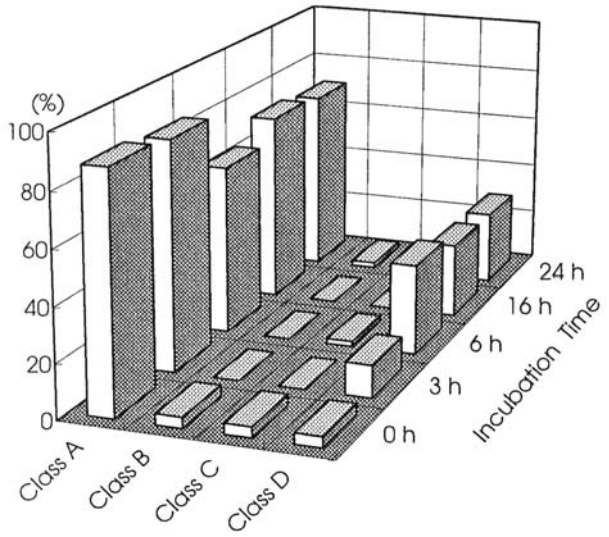

Figure 5. *Population of PC12 cell classified as A to D after cells were starved for the indicated times.*

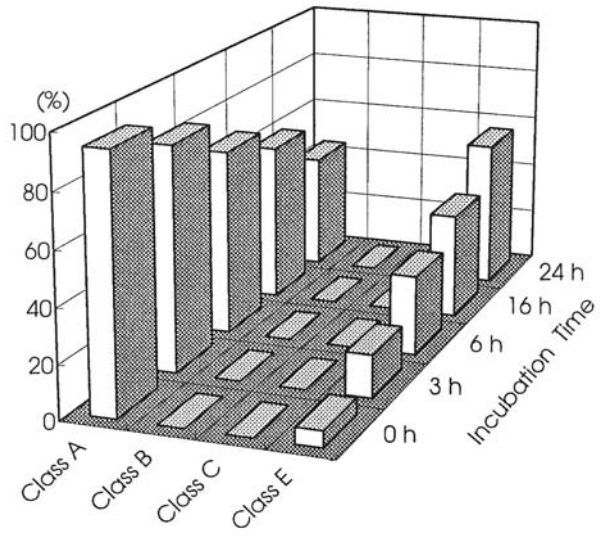

Figure 6. *Population of PC12 cells classified as A to C and D after cells were starved for the indicated times. The Comet assay was carried out without electrophoresis.*

population of cells in class D increased with time up to 6 h, then slightly decreased with further starvation. When the Comet assay was performed without electrophoresis, very clear results were obtained; only cells in class A (only the nucleus was seen) and class E were observed, and the population of cells in class E increased while that in class A decreased with ongoing starvation (Figure 6). Results for cells treated with MNU for 24 h are shown in Figure 7. Cells in class B became most numerous with 2 mM MNU treatment, while cells in class C increased with increasing MNU doses, reaching their highest level with the 4 mM dose. Cells in class D increased with an increasing dose, however, their numbers were very low. In the case of MNU-treated cells, when the Comet assay was carried out without electrophoresis, it was difficult to distinguish cells in class E from cells in other classes. Therefore, for detection of apoptotic cells, the Comet assay with electrophoresis is preferred.

Using this system, induction of apoptosis by APP^+ and APTP was analyzed together with induction by MPP^+. Figure 8 shows the results obtained with PC12 cells treated with MPP^+, APP^+ and APTP for 24 h. In every case, only the population of cells in class D increased with an increasing dose. For APTP, a high dose was required to give a population similar to that of cells treated with MPP^+ and APP^+. These results corresponded to those of our previous report showing that the cytotoxic effect of APTP on PC12 cells is weaker than that of APP^+ using a shorter treatment time (2).

For induction of apoptosis by MPP^+, APP^+ and APTP, other detection methods, such as flow cytometric analysis,

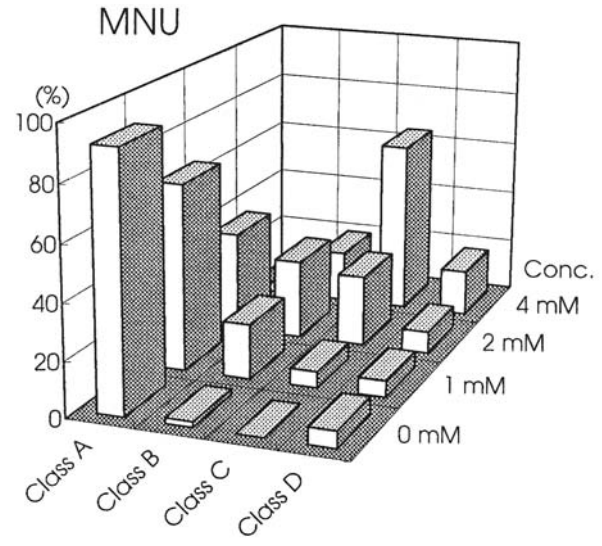

Figure 7. *Population of PC12 cells classified as A to D after cells were treated with MNU (1, 2, and 4 mM) for 24 h.*

were also applied. After cells had been treated with APP^+ or APTP at doses of 0.125, 0.5 and 1 mM, induction of apoptotic cell death was analyzed by flow cytometry. Two methods were used; analyses of cells i) in the sub-G1 region using propidium iodide and ii) in early apoptosis using an Annexin V-FITC kit. As a positive control, MPP^+ was also

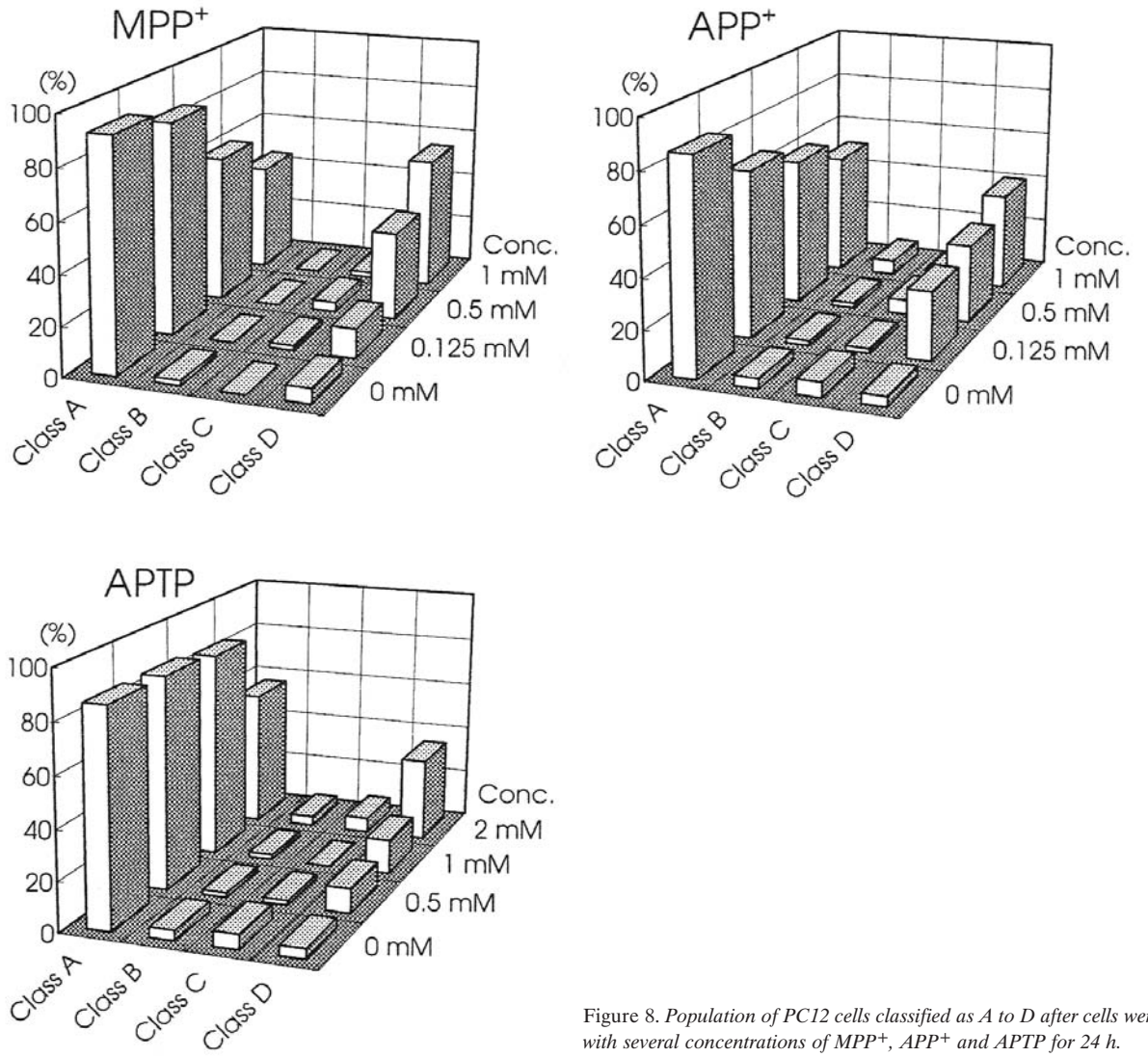

Figure 8. *Population of PC12 cells classified as A to D after cells were treated with several concentrations of MPP+, APP+ and APTP for 24 h.*

used. Flow cytometric data for the sub-G1 region after 48 h of treatment are shown in Figure 9. The percentages of cells in the sub-G1 region were calculated and summarized in Table I. With the MPP^+ treatment, the fractions of cells in the sub-G1 region increased with an increasing dose. Results of APP^+ and APTP were quite similar to those for MPP^+. This indicates that induction of apoptosis occurs with APP^+ and APTP as it does with MPP^+. Flow cytometric data on cells in early apoptosis after 30-h treatment are shown in Figure 10. With MPP^+, the cell population shifted from the lower left (LL) to lower right (LR), the state of early apoptosis, with an increasing treatment dose, and it then shifted further to the upper right (UR), the state of late apoptosis. The cell population shifted similarly with APP^+ and APTP. Cell populations in LL, LR and UR quadrants at each dose are summarized in Table II. With APP^+, the population shifted from LL to LR with an increasing dose, however, it required a very high dose to give a cytometric pattern similar to that of APTP. These results corresponded to those of our previous report showing that the cytotoxicity of APTP towards PC12 cells is stronger than that of APP^+ with a longer treatment time (2). From these results, we concluded that both APP^+ and APTP induce apoptosis in PC12 cells as does MPP^+.

In the Comet assay, apoptotic cells could be detected at 24 h after cells were treated with MPP^+, APP^+ or APTP as shown in Figure 8, while with flow cytometric analysis using an Annexin V-FITC kit, early apoptotic cells were observed

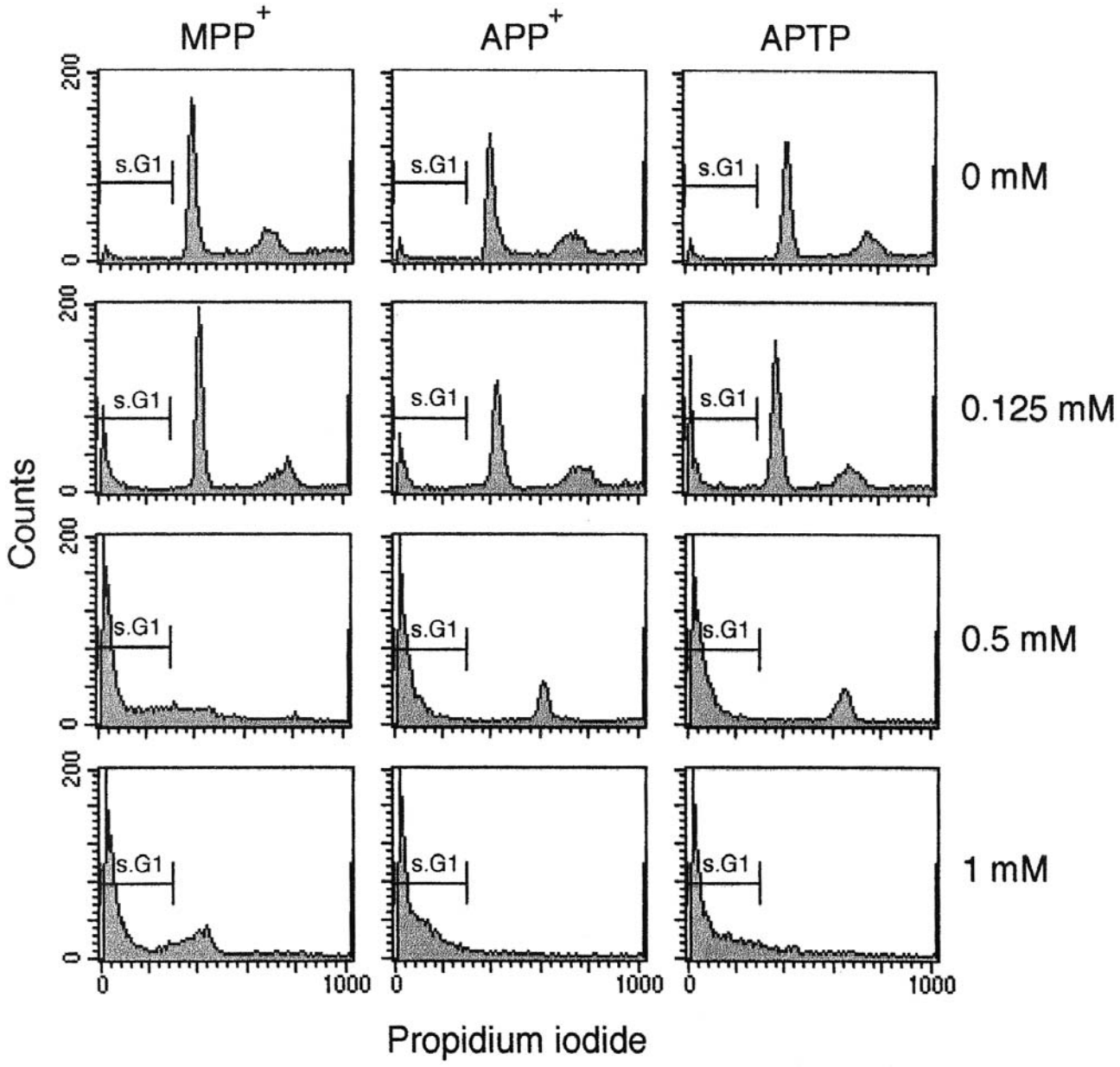

Figure 9. *Flow cytometry of PC12 cells treated with several concentrations of MPP+, APP+ and APTP for 48 h. Detection of cells in the sub-G1 region. s.G1 shown in figure denotes the sub-G1 region.*

only in the case of MPP+. For APP+ and APTP, early apoptotic cells were not observed at 24 h of treatment but did appear after 30 h, as shown in Figure 10. These results indicated that the Comet assay is a more sensitive method for detecting cells in early apoptosis than is flow cytmetric analysis using an Annexin V-FITC kit.

Apoptosis, programmed cell death, can be detected by several methods such as flow cytometric analysis (11), agarose gel electrophoresis (15), the TUNEL assay (18) and others. The Comet assay is also known to be a convenient

Table I. *Fraction of cells in the sub-G1 region*.*

Conc (μM)	% MPP+	APP+	APTP
0	1.0	1.7	1.8
125	12.4	10.8	14.1
500	75.4	61.2	65.1
1,000	66.7	84.6	77.1

*Cells were treated with MPP+, APP+ or APTP for 48h

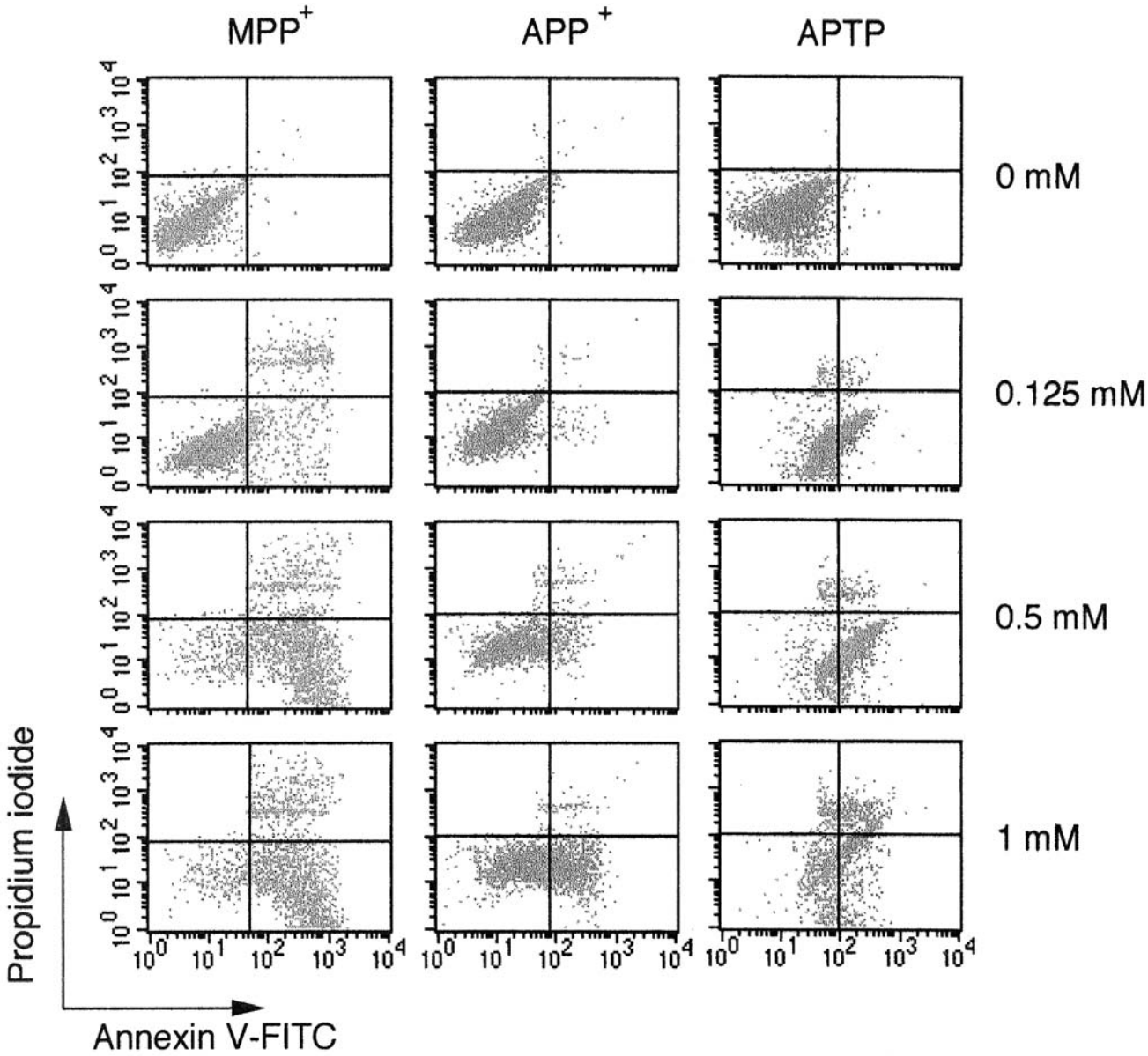

Figure 10. *Detection of cells in the early apoptotic state by flow cytometry. Cells were treated with several concentrations of MPP^+, APP^+ and APTP for 30 h.*

method for detecting DNA degradation including apoptosis (5, 6), however, very few reports have been published on DNA damage of PC12 cells. Furthermore, no studies have appeared on the detection of apoptosis in PC12 cells using the Comet assay. We modified the Comet assay to establish the procedures for determining apoptosis in these cells. Since PC12 cells are used widely as models of nerve cells, our method for performing the Comet assay should prove to be convenient for distinguishing and quantifying both apoptotic and necrotic cells.

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Table II. *Cells in LL, LR and UR quadrants*.*

Conc (μM)		% MPP+	APP+	APTP
0	LL	98.4	98.2	97.5
	LR	0.4	0.9	1.2
	UR	0.4	0.6	0.2
125	LL	77.3	95.7	67.3
	LR	10.8	2.2	32.5
	UR	10.8	0.7	0.2
500	LL	15.4	79.0	36.3
	LR	62.3	11.4	62.9
	UR	20.0	4.8	0.8
1,000	LL	12.0	49.4	45.1
	LR	56.2	43.9	34.1
	UR	25.0	4.3	20.8

* Cells were treated with MPP+, APP+ or APTP for 30 h, and flow cytometry was carried out using Annexin V and propidium iodide staining. LL; lower left, LR; lower right, UR; upper right

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