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# Dopamine-induced programmed cell death in mouse thymocytes

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**Abstract**

Exposure of mouse thymocytes to dopamine caused apoptosis (programmed cell death). This was manifested by cellular condensation and membrane damage shown by flow cytometry measurements and scanning electron microscopic study. Dopamine also affected thymocytic nuclei and their genomic DNA integrity. Most of the DNA molecules accumulated in a subdiploid peak in flow cytometry analysis, indicating DNA fragmentation to small particles. DNA analysis showed the typical pattern of 'DNA ladder' caused by internucleosomal DNA cleavage. X-ray microanalysis of the cellular elements of dopamine-treated cells showed elevation of sodium (Na), chloride (Cl) and calcium (Ca) peaks, accompanied by reduction in phosphate (P) concentrations. Comparison of the potassium (K) and P concentrations showed significant differences between the two major death processes: necrosis (induced by exposure to sodium azide ($NaN_3$)) and apoptosis (induced by dopamine). High concentrations of K indicated cell viability while reductions in P and elevations in Ca levels were found to be typical of apoptotic cell death. The antioxidant dithiothreitol (DTT) suppressed dopamine-induced apoptosis in thymocytes, suggesting that its toxicity may be mediated via generation of reactive oxygen radicals. Our study suggests that under certain circumstances, dopamine and/or its metabolites, may induce a process of apoptotic cell death of the dopamine-producing cells in the substantia nigra. Increased accessibility of dopamine to the nigral cell nucleus or inability to scavenge excess free radicals generated from dopamine oxidation triggering programmed cell death, may cause the progressive nigral degeneration in Parkinson's disease.

*Keywords:* Dopamine; Thymocyte; Programmed cell death; Apoptosis; Flow cytometry; X-ray microanalysis

## 1. Introduction

The cause for the rather selective degeneration of the substantia nigra pigmented dopaminergic neurons in Parkinson's disease is still unknown [1]. Dopamine, the natural neurotransmitter in the nigrostriatal projection, has potent anticancer properties and is highly toxic to several cell lines (e.g., melanoma) in vitro [2]. Auto-and enzymatic oxidation of dopamine generate a variety of cytotoxic oxygen radical species [3]. It is theoretically possible that nigral cell death in Parkinson's disease is etiologically linked to excessive local oxidant stress due to breakdown of dopamine, mainly by monoamine oxidase, and linked to presence of high concentrations of iron and synthesis of neuromelanin [4]. It was suggested that the formed free radicals cause membranal lipid peroxidation and rupture, leading to neuronal disintegration [5]. However, nigral degeneration in Parkinson's disease is a protracted 'silent' process that lacks the features of necrotic cell death [6].

Apoptosis is a death process with distinctive morphological characteristics set in motion by activation of a dormant nuclear genetic program for cellular self-destruction [7]. Many anticancer drugs work by triggering programmed cell death [8]. We hypothesized that dopamine itself or the reactive oxygen radicals generated during its metabolism may, under certain circumstances, cause inappropriate activation of apoptosis-like programmed cell death in substantia nigra neurons, thus leading to the evolution of Parkinson's disease. Thymocytes exposed to dexamethasone represent a well-established model for apoptosis [9]. We therefore examined whether dopamine

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can induce the typical features of programmed cell death in mouse thymocytes.

## 2. Materials and methods

### 2.1. Cell culture

Thymocytes were obtained from 4-week-old BALB/c mice. Animals were sacrificed by cervical dislocation and the thymus removed and placed in RPMI 1660 medium. The cell suspension was washed and adjusted to a concentration of $2 \times 10^6$ cell/ml in RPMI 1660 medium supplemented with 5% fetal calf serum (FCS) and 10 mM Hepes buffer. Aliquots of 2 ml thymocytes were incubated at either 37°C or 4°C with various drugs (dopamine, dexamethasone and sodium azide). The cells were washed with PBS (10 mM sodium phosphate [pH 7.4], 2.5 mM KCl, 137 mM NaCl) and processed.

### 2.2. DNA labeling and flow cytometry analysis

Thymocytes were washed twice with PBS by centrifugation for 10 min at $200 \times g$. Cell pellets were gently resuspended in PBS, at a concentration of $10^6$, and the forward scatter (FSc) and the side scatter (SSc) were simultaneously measured. Cell nuclei were isolated by resuspending cell pellets in 1.5 ml hypotonic fluorochrome solution containing propidium iodide (PI) 50 ug/ml in 0.1% sodium citrate plus 0.1% Triton X-100 (Sigma, Israel), in $12 \times 75$ polypropylene tubes (Becton and Dickinson, [BD]). The tubes were placed in the dark overnight at 4°C before the flow cytometric analysis. Cell nuclei were evaluated for DNA fragmentation by using flow cytometry (BD) as described by Nicoletti et al. [11]. Argon laser beam (488 nM) was used to excite the PI dye and the red fluorescence was collected via a 610 nm long pass filter. The data was processed by a Hewlett Packard computer and analyzed with Lysis software (BD).

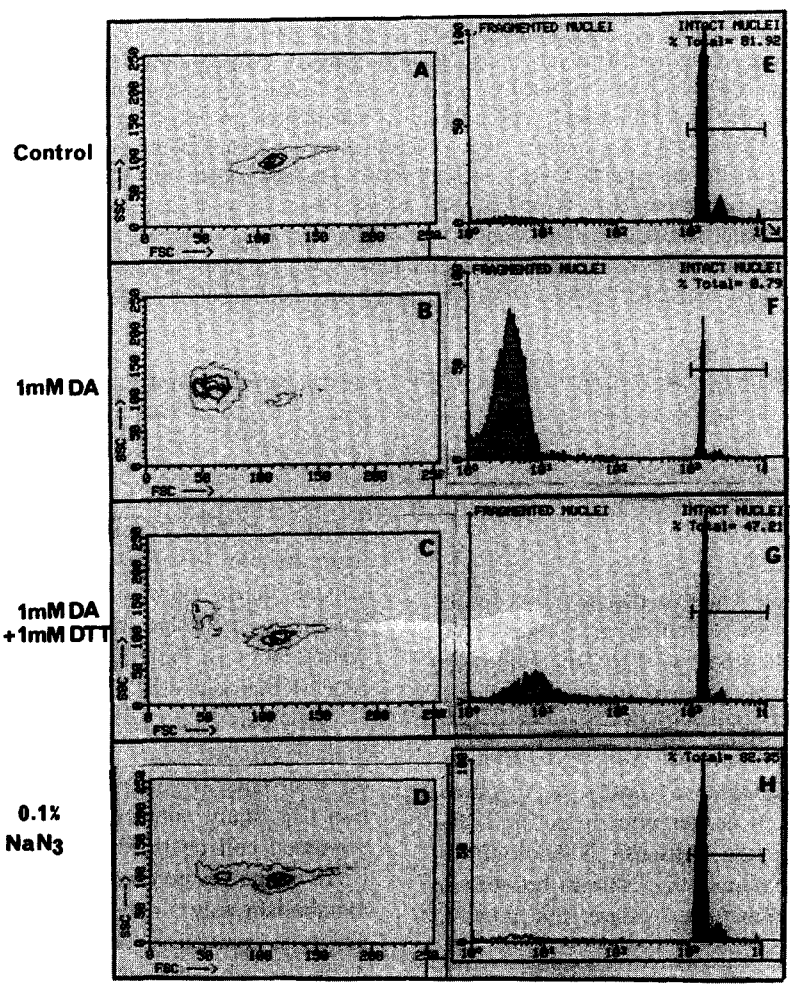



Fig. 1. Flow analysis of dopamine-treated mouse thymocytes. Cells were treated for 3 h at 37°C with dopamine (D,F), dopamine (on line) with dithiothreitol (DTT) (C,G) or sodium azide (NaN3) (D,H). Left panels: whole cell analysis for cell size (FSC, forward high scattering) and cell granulation (SSC, side high scattering). Right panels: estimate of DNA content in isolated cell nuclei.

### 2.3. X-ray microanalysis of cells

X-ray microanalysis (XRMA) for measurement of cellular elements, including sodium (Na), phosphate (P), sulphate (S), chloride (Cl), potassium (K) and calcium (Ca), was performed on mouse thymocytes after 3 h of treatment with the various drugs. The cells were rinsed, resuspended in PBS and attached to Thermanox (Nunc, Nupervile, IL) plastic coverslips which had been previously treated with poly-L-lysine (Sigma, Israel). The cell monolayers on the coverslips were placed in multiwell dishes filled with buffer. The attached cells were quickly rinsed by dipping in ammonium acetate 0.15 M (pH 7.0), for 2–3 s. The rinsed cells were immediately frozen with liquid nitrogen, freeze-dried at −80°C and than coated with a layer of carbon [10]. XRAM was performed on 50 cells from each sample using an eXL-Link system (Oxford Institute, Oxford, UK) attached to a Jeol 840 scanning electron microscope (S.E.M.).

### 2.4. DNA analysis

DNA was isolated from the cells, washed twice with PBS and pelleted by centrifugation at 200 × *g* for 5 min at room temperature. Cell pellets were resuspended at a concentration of $2 \times 10^7$ cells/ml in cell lysis buffer (10 mM EDTA, 10 mM Tris (pH 7.4), 0.25% Triton X-100 and 50 μg/ml proteinase K) and incubated for 1 h at 50°C. RNAse A was then added at a concentration of 0.01 mg/ml and incubation at 37°C was continued for 1 h. The crude DNA preparations were then extracted with phenol, and (twice) with chloroform/isoamyl alcohol (24:1). The DNA was precipitated for 24 h in 2 volumes of ethanol at −70°C, and then resuspended in 10 mM Tris-HCl (pH 8.0) 1 mM EDTA (TE buffer). Electrophoresis of the isolated DNA was carried out in 2% agarose gels. Before electrophoresis, loading buffer (10 mM EDTA, 0.25% (W/V) bromophenol blue and 50% (V/V) glycerol) was added to each sample at a 1:5 ratio. Samples were then heated to 65°C for 10 min and plunged into ice. Approximately 10 μg of DNA was loaded into each well and electrophoresis was carried out at 6V per cm of gel in TBE buffer (2 mM EDTA [pH 8.0], 89 mM Tris, 89 mM boric acid). An *Hea*III digest of OX-174 DNA was applied to each gel to provide size markers of 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 base pair (bp), respectively.

*Scanning electron microscopy (SEM) of thymocytes.* The cells, fixed with glutaraldehyde/paraformaldehyde, were prepared for SEM by the triple fixation GTGO method [12]. Briefly, cells were post-fixed in 2% $O_sO_4$, then fixed again with 2% tannic acid/guanidine, and dehydrated in graded ethanol solutions; ethanol was then exchanged with graded solutions of Freon 113, and the samples were air-dried and gold-coated. The cells were examined with a Joel 840 scanning electron microscope; 200 cells from each sample were visualized for cell morphology [12].

## 3. Results

### 3.1. Flow cytometric pattern of dopamine-treated thymocytes

Whole thymocyte cells were first analyzed by light scatter flow cytometry. The SSc/FSc that might indicate cell size parameter and SSc that serves as a measure for cell granularity were determined simultaneously. The untreated control thymocyte population showed a uniform pattern with minor differences in cell size (Fig. 1A). Exposure of the cells to physiological concentrations of dopamine (0.3–1 mM) for 3 h caused a significant shift to the left, indicating that most of the cells underwent cellular condensation. Similar results were observed using dexamethasone which is known to induce apoptosis in the model system. In addition to the left shift changes, the cell population showed an increased SSc parameter (Fig. 1B). The addition of the antioxidant dithiothreitol (DTT) (1

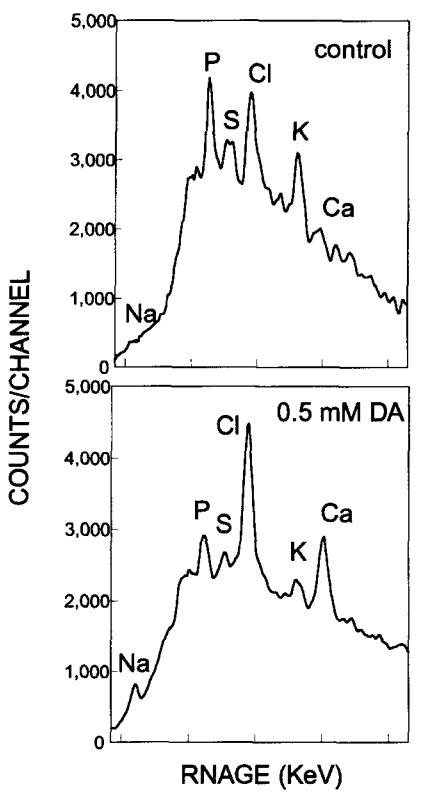



Fig. 2. X-ray microanalysis spectra of thymocytes treated with dopamine (0.5 mM) in comparison to control, untreated cells. At the end of the dopamine treatment, cells were attached to Thermanox coverslips using poly-L-lysine, rinsed for 2 s in ammonium acetate, immediately fixed in liquid nitrogen, carbon-coated and elemental composition was determined by SEM with an X-ray microprobe. Each spectrum was accumulated from 50 individual cells.

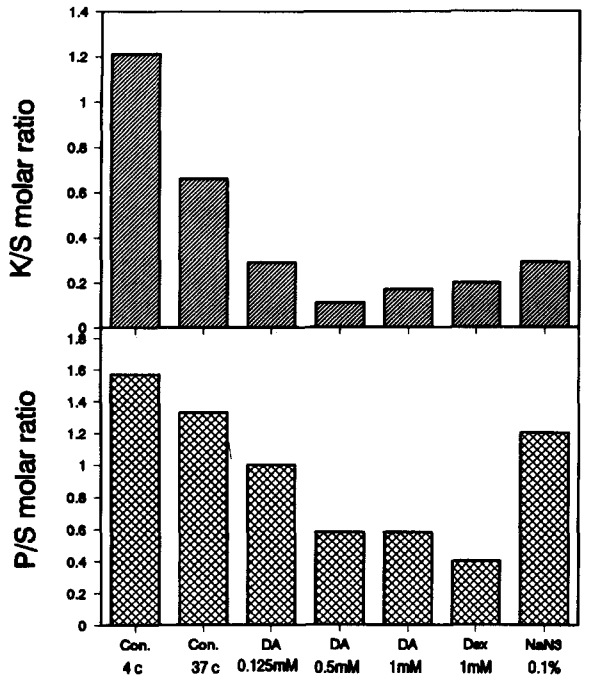



Fig. 3. The K/S and P/S molar ratios of thymocytes exposed to dopamine, dexamethasone or sodium azide (NaN3). The cells from three different experiments were treated as in Fig. 2 and the integrated peak ratios obtained from 50 cells were calculated. Data from three different experiments are the mean ± S.D. The group data were compared by two-tailed *t*-test ( * $P < 0.05$, * * $P < 0.01$ as compared to controls 37°C).

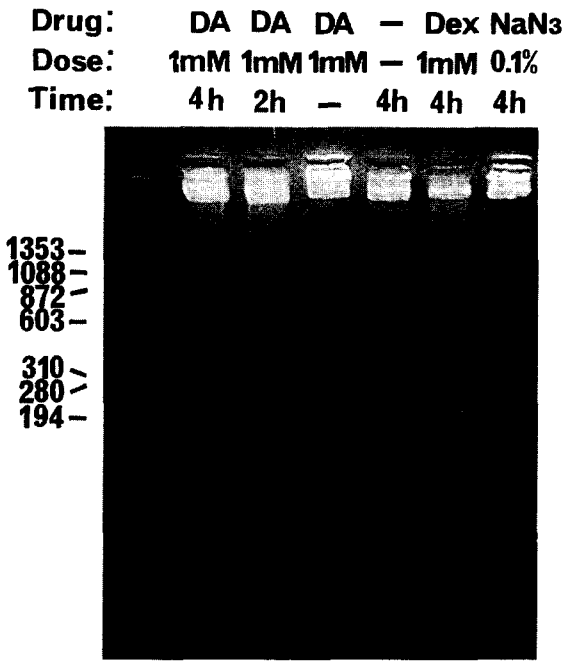



Fig. 4. DNA analysis of dopamine-treated mouse thymocytes. Cells were treated with dopamine, dexamethasone (Dex) or sodium azide (NaN3) as indicated. The isolated DNA was analyzed on agarose gel-electrophoresis and DNA from phage x174 cut with *Hae*III was used as a molecular weight marker.

mM) to the dopamine-treated cells inhibited most of the observed effects (Fig. 1C). Thymocytes were exposed to 0.1% sodium azide ($NaN_3$) for 3 h to provide a model of necrotic cell death. Although $NaN_3$ caused a massive cell death, as indicated by trypan blue exclusion assay (data not shown), most of the morphological changes seen with dopamine were not observed following exposure to this drug (Fig. 1D).

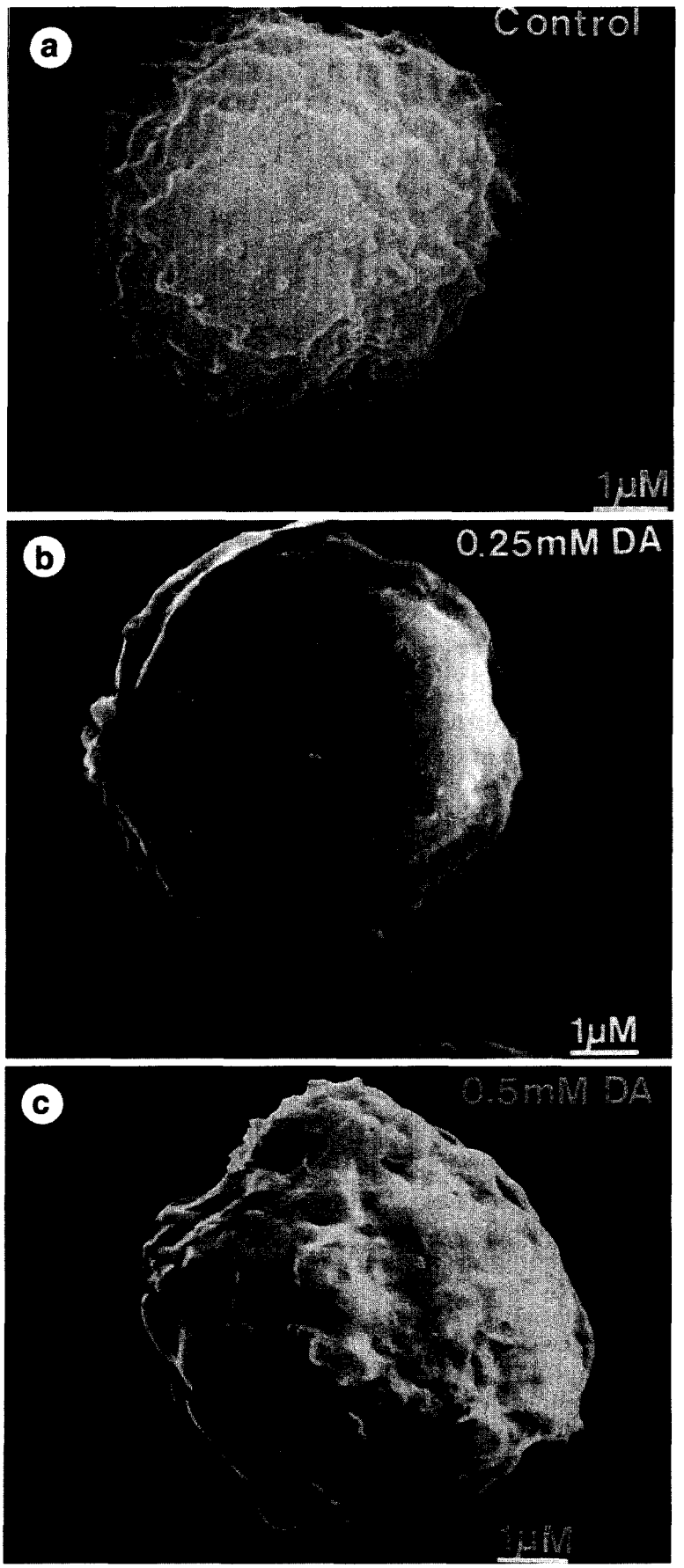
Analysis of the propidium-iodide-stained DNA by flow cytometry enabled the estimation of DNA content of isolated nuclei. In untreated cells, 82% of the measured events created a sharp, distinct peak of intact DNA, characteristic of the diploid form (Fig. 1E). Exposure to 1 mM dopamine caused a marked reduction of the DNA content in the diploid peak to 8.8% and most of the events created a typical 'apoptotic' subdiploid peak (Fig. 1F). Combined treatment with DTT (1 mM) provided protection against DNA fragmentation with about 40% of the events present in the diploid peak (Fig. 1G). In contrast, necrotic cell death caused by $NaN_3$ was not accompanied by DNA fragmentation as there was no accumulation of a subdiploid peak (Fig. 1H).

### 3.2. X-ray microanalysis of thymocytes

X-ray microanalysis of cells provides an accurate estimate of the concentrations and the molar ratios of the cellular elements, such as K, Na, P, Cl, and S [10]. Thymocytes, treated by the various drugs, were submitted to XRMA. The spectra shown in Fig. 2 represent data accumulated from a sample of 50 cells, measured simultaneously. Fig. 2 shows that in dopamine-treated cells, there is a decrease in K and, in parallel, an increase in the levels of Cl, Na and Ca in comparison to the control cells. These results may reflect membranal damage that leads to K leakage out of the cells and influx of Na, Cl and Ca influx. The S peak is generated mostly by the sulphuric residues of cellular proteins. Since the concentration of such proteins is relatively stable (compared to the ions that can easily leak out through the membrane), we used the S peak as an internal standard. A decrease in the K/S molar ratio is indicative of cell death [10], while a low P/S molar ratio may reflect DNA breakdown and nucleotide leakage out of the cells.

Fig. 3 shows the molar ratios of K/S and P/S obtained during the various treatments of the thymocytes. Cells

Fig. 5. Scanning electron microscopy (SEM) showing the morphological alterations of dopamine-treated thymocytes. Control, untreated cells have well-preserved microvilli and some outer membrane ruffles (a), while the dopamine-treated cells are completely smooth or shrunk following exposure to 0.25 mM dopamine (b). Exposure to higher concentrations of dopamine causes cell shrinkage and membrane disintegration (c). Original magnification ×5000.


a
Control
1μM
b
0.25mM DA
1μM
c
0.5mM DA
1μM

treated either by dopamine, dexamethasone or $NaN_3$ showed a decrease in total cellular K per cellular protein (K/S). These parameters indicate inviability of those cells after 3 h of exposure to these drugs. No significant differences were measured between dopamine, dexamethasone and $NaN_3$. In contrast, the P/S molar ratios showed marked and significant differences in the dopamine and dexamethasone-treated cells as compared to those treated with $NaN_3$. The similar alterations of the P/S molar ratios in the thymocytes exposed to dopamine and dexamethasone is unique and points to a similar death mechanism involving breakage and extracellular leakage of small DNA fragments.

### *3.3. Dopamine induces DNA fragmentation in thymocytes*

One of the features distinguishing apoptosis from necrosis is the early onset of specific endonuclease-mediated cleavage of cellular DNA into nucleosome ladders [13]. We therefore examined whether dopamine can elicit a similar pattern of DNA fragmentation in thymocytes. DNA analysis of cells exposed to 1 mM dopamine revealed the typical DNA cleavage already after 2 h of treatment (Fig. 4). Since untreated thymocytes may often undergo spontaneous DNA fragmentation during incubation at 37°C both temperatures of 4°C and 37°C incubation conditions were used as controls. Indeed, some DNA fragmentation was seen in untreated cells but it is markedly different from the pattern observed following dopamine treatment for 4 or even 2 h. The fragments seen in Fig. 4 were formed in order of sizes, i.e., 180, 360, 540 and 720 bp etc., creating the characteristic apoptotic DNA ladder. The similar DNA fragmentation pattern seen in cells treated with dopamine and dexamethasone in contrast to $NaN_3$ suggests that the cell death mechanism induced by dopamine is due to apoptosis. DTT prevented the dopamine-induced formation of the typical apoptotic 'DNA ladder' on agarose gel (data not shown), similar to its inhibitory effect on DNA fragmentation seen in the FACS analysis (Fig. 1).

### *3.4. Dopamine-induced ultrastructural features of apoptotic death*

Cells undergoing programmed cell death shrink and their outer membranes form bullous structures termed blebs [14]. SEM of mouse thymocytes treated with dopamine showed various cellular alterations (Fig. 5a–c). Exposure for 3 h to 0.25 mM dopamine caused marked membranal changes. The outer membrane totally disappeared with the characteristic formation of blebbing. Fig. 5b shows that some of these blebs are detached from the plasma membrane. Exposure to a higher concentration of dopamine (0.5 mM for 3 h) caused the beginning of cell shrinkage and marked plasma membrane disintegration (Fig. 5c). Thymocytes treated with DEX showed similar apoptotic features to those observed following exposure to dopamine. Treatment with DTT inhibited the morphological alterations induced by dopamine (data not shown).

## 4. Discussion

We exposed thymocytes to dopamine, at concentrations of 0.1–1 mM, similar to the physiological levels within the nigral neurons, as shown by Michel and Hefti [4]. Flow cytometry measurements on thymocytes treated with dopamine (1 mM, for 4 h) demonstrated condensation and granulation of whole cells and accumulation of a subdiploid peak of fragmented DNA particles (Fig. 1). DNA analysis revealed a distinct laddering pattern of internucleosomal DNA cleavage to discrete fragments, differing in size by multiples of about 180 base-pairs (Fig. 4). Furthermore, electronmicroscopy demonstrated that thymocytes exposed to dopamine undergo marked morphological changes, including creation of outer membranal blebs (Fig. 5). These alterations were identical to those caused by treatment with dexamethasone, a classical inducer of apoptosis in thymocytes [15]. By contrast, exposure to $NaN_3$, a toxin that typically kills cells by causing necrosis [16], did not induce any of the above markers for apoptosis in thymocytes.

We also carried out X-ray microanalysis of dopamine-treated and control thymocytes to estimate spectra of various intracellular elements. It was demonstrated that increased peaks of K, P and Cl may provide an index for healthy, well-preserved cells [10]. In contrast, we found relatively elevated Na, Cl and Ca and reduced P peaks in thymocytes exposed to dopamine (Fig. 2). Such alterations in concentrations of the ions are generally indicative of membranal damage [17]. The increased K concentration might be a result of inactivation of the K leak-out channels [18], while elevated levels of Na and Cl might reflect dysfunction of the Na + /K + ATPase [17]. The reduction in the P peak may be due to extracellular leakage of small DNA fragments and nucleotides through defects created in the outer membranes of the cells undergoing apoptotic alterations. The stability of the S peak during programmed cell death might be explained by the fact that the sulphur-containing proteins are much larger than the fragmented DNA particles and, therefore, do not leak out of the cells and their levels remain unaltered intracellularly. In addition, the X-ray spectra analysis showed marked Ca influx into the dopamine-treated thymocytes (Fig. 2). Changes in the intracellular soluble Ca have been reported in association with apoptosis [16]. Our study is the first to report direct estimation of the alterations that occur in total (bound and free) cellular Ca during programmed cell death.

Since cellular protein concentrations, represented by the S peak, were found to be relatively stable (Fig. 2); we have used this peak as an internal standard to follow spectra alterations. Comparison of K peaks in treated and un-

treated cells shows a significant correlation between cell viability and high intracellular levels of K. Treatment with low-dose dopamine (0.125 mM), dexamethasone or $NaN_3$ show influx of K. In contrast, influx of P can be seen only in cells treated with 0.5 mM dopamine and dexamethasone, while the levels of P in cells treated with $NaN_3$ have not changed. The low P levels found in cells treated with dopamine and dexamethasone are consistent with our other experiments showing DNA fragmentation. Therefore, these findings suggest that XRMA can be used as a very powerful tool in the evaluation of cells undergoing apoptosis.

We have recently shown that dopamine is also capable of inducing apoptosis in cultured chick sympathetic neurons [19] with features similar to those observed following nerve growth factor (NGF) deprivation [20]. The mechanism through which dopamine sets in motion programmed cell death in thymocytes and sympathetic neurons is unknown. The responsible toxic agent may be dopamine itself and/or one or more of its metabolic by-products, such as semiquinones, quinones, neuromelanin and reactive oxygen radical species, e.g., $H_2O_2$. It was recently shown that free radicals may be involved in triggering programmed cell death [21]. Our observations that combined treatment with the antioxidant DTT prevented apoptosis in thymocytes exposed to dopamine (Fig. 1). DTT can also inhibit the spontaneous, non-enzymatic conversion of dopamine to dark dopamine-neuromelanin in cell-free culture media (Offen et al., in preparation). These observations suggest that the apoptosis-inducing effect of dopamine shown in our experiments is mainly via its autooxidation metabolites.

Since receptors for dopamine (but not the dopamine uptake machinery) are present on thymocytes, we cannot exclude the possibility that the observed dopamine toxicity is receptor-mediated. However, we think that receptor involvement is not essential. Dopamine receptors recognize and bind intact dopamine, while the toxicity, as mentioned above, is probably mediated through its oxidative metabolites.

Based on the above, we propose that the anticancer effect of dopamine seen particularly in melanoma cells [22] may be due to induction of programmed cell death via a similar mechanism.

There is a gradual decline in the number of substantia nigra dopaminergic neurons during normal aging [23]. The cause for this seemingly 'physiological' neuronal loss is unknown but it is not unlikely that it is due to activation of programmed cell death in 'selected' individual neurons. Since the first signs of Parkinson's disease emerge only after loss of about 70–80% of the dopaminergic neurons [24], the death of nigral cells during aging usually remains clinically silent. It is feasible that under normal circumstances nigral neurons are equipped with adequate mechanisms (e.g., vesiculation of dopamine and naturally-occurring intracellular enzyme systems for the scavenging of excess free radicals) to restrain the potentially toxic effects of their own neurotransmitter, dopamine. However, it may be hypothesized that if in Parkinson's disease there is a yet unidentified failure of one or more of such defense mechanisms, enabling dopamine or its toxic products to gain access to the unprotected nucleus, programmed cell death may be triggered and the nigral neuron will self-destruct.

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