Effect of polyamine depletion on chromatin structure in U-87 MG human brain tumour cells

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The chromatin structure of polyamine-depleted U-87 MG human brain tumour cells was studied by following the kinetics of digestion of cell nuclei by micrococcal nuclease and bovine pancreatic DNAase I. Cells growing in monolayers were treated with either α -difluoromethylornithine (DFMO), to deplete putrescine and spermidine, or N^1,N^{14} -bis(ethyl)-homospermine (BE-4-4-4), to deplete putrescine, spermidine and spermine. BE-4-4-4 increased the initial rates of digestion and the magnitudes of limit digest by both enzymes; DFMO increased the limit digests without affecting initial digestion rates. Addition of 1 mM-putrescine 1 day after addition of DFMO reversed the effect of DFMO on limit digests. (Because polyamine uptake is low in cells treated with BE-4-4-4, and because putrescine does not reverse the growth-inhibitory effects of BE-4-4-4, reversal of the effects of BE-4-4-4 with putrescine was not attempted.) The increases in initial rates and limit digests did not result from changes in the lengths of nucleosomal or linker DNA, from blocks in cell-cycle progression, or from growth inhibition caused by DFMO or BE-4-4-4. Thus, because the limit digest is highest in cells with the lowest polyamine levels, it seems clear that the enhanced enzymic digestion of nuclei is caused by polyamine depletion and its possible effect on chromatin structure.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are aliphatic cations present in all mammalian cells. Although these compounds are important to the growth and differentiation of cells [1], the molecular mechanisms of their biological functions have not been completely elucidated. During the past decade, researchers have studied these mechanisms by using agents that deplete intracellular levels of polyamines. One such agent, α -difluoromethylornithine (DFMO), is an inhibitor of polyamine biosynthesis that has been used widely both clinically and in laboratory experiments [2]. In most cell lines, DFMO depletes putrescine and spermidine, but not spermine. Although recently developed polyamine analogues can deplete all three cellular polyamines [3], many are ineffective as cell-growth inhibitors. This may result from an ability of the analogue to mimic biological functions of polyamines, possibly by interacting with cellular polyamine-binding sites [4,5].

Negatively charged nucleic acids are among the probable intracellular sites to which cationic polyamines bind. Spermidine and spermine condense and aggregate DNA and induce B–Z and B–A transitions *in vitro* [6–8]. Computer-graphics modelling and physico-chemical studies indicate that spermine can induce bends in certain DNA sequences, possibly by forming hydrogen bonds with nucleotides in specific positions before the onset of condensation and aggregation [9–13]. These results, combined with the growth inhibition caused by some spermine analogues, lead us to propose that the ability of those analogues to aggregate DNA *in vitro* plays an important role in growth and survival *in vivo* [14,15]. From this standpoint, the spermine analogue N^1,N^{14} -bis(ethyl)homospermine (BE-4-4-4) is of particular interest. This analogue is much weaker than spermine in bending

and aggregating DNA [14], and it enters cells and inhibits their growth [15,16].

The DNA-condensing property of polyamines may be important as DNA compacts into chromatin during mitosis. Because the positioning of nucleosomes and the transcriptional state of chromatin may be important in the process of condensation, we used micrococcal nuclease (MNase), which selectively digests the linker regions between nucleosomes in eukaryotic chromatin [17–19], and bovine pancreatic DNAase I, which acts specifically either on transcriptionally active regions of chromatin or on sites flanking those regions [20–22], as probes to study effects of DFMO and BE-4-4-4 on chromatin structure. It is generally agreed that both MNase and DNAase I follow complex reversible Michaelis–Menten kinetics involving marked substrate and product inhibition [19,23,24]. Both enzymes are sensitive to structural changes in DNA and prefer relaxed DNA to condensed DNA [25,26]. In addition, DNAase I is specific for right-handed B-DNA and does not digest left-handed Z-DNA [27,28].

Here we report DNAase I and MNase digestion kinetics in nuclei isolated from human brain tumour cells previously treated with DFMO or BE-4-4-4. The kinetics of digestion of nuclei from serum-deprived cells and from cells in which the effect of DFMO was reversed by putrescine are also reported.

MATERIALS AND METHODS

Materials

DFMO was generously provided by the Marion–Merrell Dow Research Institute, and BE-4-4-4 was given by Professor Raymond J. Bergeron, Department of Medicinal Chemistry, University of Florida, Gainesville, FL, U.S.A. Bovine pancreatic DNAase I (EC 3.1.21.1; Boehringer), staphylococcal nuclease

Abbreviations used: DFMO, α -difluoromethylornithine; BE-4-4-4, N^1,N^{14} -bis(ethyl)homospermine; MNase, micrococcal nuclease; PI, propidium iodide; PBS, phosphate-buffered saline.

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(MNase) (EC 3.1.31.1; Pharmacia LKB Biotechnology), calf thymus DNA, RNAase A (EC 3.1.27.5; Sigma Chemical Co.) and proteinase K (Merck) were purchased from commercial suppliers. RNAase A was heated at 80 °C for 30 min in a water bath to destroy DNAase contaminants. Calf thymus DNA was dialysed [29] and stored in 1 mM-Tris/HCl buffer (pH 7) at -20 °C. Putrescine, spermidine, spermine (Calbiochem), phenol, chloroform (Molecular Biology Grade; International Biotechnologies, New Haven, CT, U.S.A.), 8-hydroxyquinoline, 3-methylbutan-1-ol, ethidium bromide, and propidium iodide (PI) (Sigma) were used without further purification. All other chemicals were reagent grade, and deionized distilled water was used as the solvent.

Cell culture

U-87 MG cells were grown as monolayers at 37 °C in T-175 flasks with 35 ml of minimal essential medium supplemented with non-essential amino acids and 10% (v/v) fetal-calf serum [30]. At 24 h after cell seeding, sterile solutions of DFMO (final concn. 1 mM) or BE-4-4-4 (final concn. 10 µM) were added to the medium [15]. For reversal studies, 1 mM-putrescine was added 1 day after addition of DFMO. Serum deprivation was achieved by growing cells for 3 days and then replacing the medium with Hanks' balanced salt solution.

Isolation of nuclei

Nuclei were isolated by a slight modification of a procedure described elsewhere [31]. Cells were harvested, washed twice with ice-cold phosphate-buffered saline [KH_2PO_4 (0.2 g/l), $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (2.16 g/l), KCl (0.2 g/l), NaCl (8.0 g/l), pH 7.0], and incubated on ice for 5 min in a sterile lysis buffer containing 0.25 M-sucrose, 0.06 M-KCl, 0.05 M-NaCl, 0.01 M-Mes, 0.01 M-MgCl₂, 0.001 M-CaCl₂, 0.0001 M-phenylmethanesulphonyl fluoride and 0.5% Triton X-100 (pH 6.5). Nuclei were pelleted by centrifugation at 5000 rev./min for 10 min in a Beckman JA-17 angled-head rotor in a Beckman model J2-21M refrigerated centrifuge. The pellet was washed twice with lysis buffer and once with storage buffer containing 0.005 M-MgCl₂, 0.01 M-Pipes, 0.2 M-NaCl and 0.001 M-CaCl₂ (pH 6.8), resuspended in a minimal volume of the same buffer, and stored at 4 °C. The purity of the nuclear preparation was checked by u.v.-absorption spectra [19] and fluorescence microscopy after staining with ethidium bromide. The concentration of DNA in the suspension of nuclei was determined from the A_{260} in 1.0 M-NaOH [32] and was corrected for pH effect [33].

Enzyme digestion

DNAase I digestion was carried out in the storage buffer. The buffer used for the MNase digestion had the same composition as the storage buffer, but the pH was adjusted to 8.6 with 1 M-NaOH. Enzyme activity was checked weekly by digestion of calf thymus DNA. The rate of digestion by MNase and by DNAase I was monitored by observing the increase in A_{260} of DNA at 37 °C. These studies were performed with a Perkin-Elmer Lambda 4C spectrophotometer equipped with an electrical heating system and coupled with an IBM AT-compatible personal computer. Softways (Moreno Valley, CA, U.S.A.) software was used for data collection, storage and analysis.

Nuclei were preincubated in assay buffer on ice for 5 min in 1.5 ml sterile silicone-treated plastic Eppendorf tubes. Ice-cold enzyme solution (0.8 unit) was added, and the volume was adjusted to 70 µl with the respective assay buffers. The tubes were heated in a water bath at 37 °C, removed after appropriate time intervals, and chilled. The reaction was stopped by adding 70 µl of ice-cold 5 mM-EDTA. In the zero-time tube, EDTA was

added before the addition of enzymes. Each sample was diluted with assay buffer to 500 µl final volume and centrifuged at 10000 rev./min for 10 min, and the supernatant was transferred to quartz cuvettes. The A_{260} was measured at room temperature, and the data were fitted to the equation

$$A_{260}^t = P1(1 - e^{-P2 \cdot t})$$

where A_{260}^t is the A_{260} at time t after the start of the reaction, and P1 and P2 are variable parameters. The initial velocity (v_0) of the enzyme reaction was then calculated from the equation

$$v_0 = (dA_{260}^t/dt)_{t=0} = P1 \cdot P2.$$

Cell cycle analysis

Approx. 1.25×10^6 cells were trypsin-treated and harvested as a single-cell suspension. Cells were pelleted at 800 rev./min for 5 min at 4 °C, washed twice with ice-cold phosphate-buffered saline, and fixed in 70% ethanol [34]. Approx. 1×10^6 fixed cells were suspended in 15 ml of PBS containing Ca^{2+} (0.1 g/l) and Mg^{2+} (0.1 g/l), centrifuged at 1000 rev./min for 10 min, and incubated for 1 h with RNAase A (final concn. 100 µg/ml) in 0.5 ml of PBS at 37 °C. A stock solution (1 mg/ml) of PI was freshly prepared in ethanol, and diluted 1:10 (v/v) with PBS. A 0.5 ml portion of this diluted PI was added to the cells, and the samples were kept in the dark at 0 °C for 30 min. The cell-cycle distribution of 10000 cells was determined with a Becton-Dickinson Facscan flow cytometer, equipped with a doublet-discriminator. The polynomial fit (SFIT) method was used for data analysis [34].

Agarose-gel electrophoresis

Nuclei (0.12 A_{260} unit/ml) from control and treated cells were digested with 0.8 unit of MNase for 6 min as described above. The digestion was stopped by adding 70 µl of lysis buffer containing 100 mM-Tris/HCl, 1.2 M-NaCl, 30 mM-EDTA and 0.4% laurylsarcosine (pH 7.5). The mixture was incubated for 1 h at 37 °C with 10 µl of RNAase A (final concn. 50 µg/ml) and then overnight at 37 °C with 10 µl of proteinase K (final concn. 500 µg/ml). Proteins were extracted twice with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) and twice with chloroform [35]. The aqueous phases containing DNA were pooled and adjusted to 0.2 M-Na⁺. The DNA was precipitated by incubating overnight at -20 °C with 3 vol. of chilled ethanol. It was then pelleted by centrifugation at 10000 rev./min for 10 min at 4 °C, dried in a vacuum desiccator, and redissolved in a minimum volume of Tris/EDTA buffer (10 mM-Tris/HCl, 1 mM-EDTA, pH 7). Electrophoresis was carried out in 1.5% agarose in 10 mM-Tris acetate buffer (pH 7) containing 1 mM EDTA for 3.5 h at 1.2 V/cm.

RESULTS AND DISCUSSION

In U-87 MG cells, intracellular putrescine and spermidine are depleted by 1 mM-DFMO, and all three cellular polyamines are depleted by 10 µM-BE-4-4-4 after 96 h of treatment ([15]; M. Sturkenboom, unpublished work). These conditions were used to treat cells with the drugs throughout these studies.

With nuclei isolated from control cells and with those from cells treated with DFMO or BE-4-4-4, digestion by MNase and DNAase I reached a plateau within 10 min of incubation (Fig. 1). The final amount of DNA digested was higher in nuclei from drug-treated cells than in nuclei from control cells. Nuclei from cells treated with BE-4-4-4 were digested more completely than those from DFMO-treated cells.

The plot of initial digestion rate (v_0) versus concentration of nuclei (Fig. 2) showed that the v_0 of both MNase (Fig. 2a) and

Fig. 1. Time course of MNase and DNAase I digestion of nuclei isolated from U-87 MG cells

The time course of digestion with (a) MNase and (b) DNAase I of cell nuclei at 37 °C ($A_{260}/\text{ml} = 0.11$) was determined by measuring the A_{260} of the supernatant in a final volume of 500 μl . Before the isolation of nuclei, the cells were treated with 1 mM-DFMO (●) or 10 μM -BE-4-4-4 (▼) for 96 h, or with 1 mM-DFMO for 96 h followed by 1 mM-putrescine (Put) for 72 h (□). Other cells were serum-deprived for 48 h (△) or used as controls (○; CON). Each data point is the average of two or three separate observations.

DNAase I (Fig. 2b) was markedly higher in nuclei from cells treated with BE-4-4-4 than in nuclei from control cells. DFMO treatment, however, had no significant effect on the initial velocity of digestion by either enzyme. Under non-aggregating conditions, spermidine, spermine or BE-4-4-4 did not affect the rate or extent of digestion of calf thymus DNA by either enzyme *in vitro* (results not shown). These data suggest that the apparent V_{max} values for enzymic digestion of nuclei from DFMO-treated cells are similar to those of control cell nuclei, whereas the apparent V_{max} values for BE-4-4-4-treated cells are greater than those for control cells. The concentration of the enzyme-recognizable substrates in the nuclei is unknown, and the low yield of nuclei from monolayer cultures restricted the number of data points (Fig. 2). For these two reasons, we could not determine the absolute kinetic parameters for either enzyme by fitting the data points shown in Fig. 2 to the complex reversible Michaelis-Menten equation [23,24].

Replenishing polyamines by adding putrescine to DFMO-treated cells decreased the magnitude of the limit digests to below control levels, as did inhibiting cell growth by serum deprivation (Figs. 1a and 1b). Because the uptake of extracellular putrescine is much lower for cells treated with BE-4-4-4 than for control cells [15], and because putrescine does not reverse the growth-inhibitory effects of BE-4-4-4 [15], the effects of added putrescine on cells treated with BE-4-4-4 were not studied.

Fig. 2. Kinetics of MNase and DNAase I digestion of nuclei isolated from U-87 MG cells treated with DFMO or BE-4-4-4

The plot of initial velocities of digestion with (a) MNase and (b) DNAase I at different concentrations of nuclei isolated from control cells (○; CON) and from cells treated with 1 mM-DFMO (▲) or 10 μM -BE-4-4-4 (■). Each data point is the average of two separate experiments.

Putrescine added to DFMO-treated cells can replenish depleted cellular polyamines to levels higher than those in control cells [5]. Furthermore, BE-4-4-4 is more effective than DFMO in depleting polyamines [15,16]. Polyamine levels are therefore highest in cells treated with both DFMO and putrescine, followed by control cells, DFMO-treated cells and BE-4-4-4-treated-cells in that order. This is the exact reverse of the order of limit digests in the four types of cell treatments (Fig. 1). Therefore, in our studies, cellular polyamine content is inversely proportional to the amount of nuclease-recognizable DNA present in nuclei. Neither polyamines nor BE-4-4-4 affected the activity of MNase or DNAase I on calf thymus DNA *in vitro*, and h.p.l.c. [36] detected no polyamines in nuclear preparations from either control or drug-treated cells (results not shown). The effects of these drugs on enzyme activity thus appear to result from a long-term effect of polyamine depletion and/or the intracellular presence of the drugs on chromatin structure, rather than from short-term variation in nuclear polyamine concentrations during enzyme digestion.

The most likely reason for the enzyme-susceptibility of nuclei from polyamine-depleted cells is a change in the state of condensation of the nucleosomes or linker DNA. This would increase the amount of uncondensed DNA, which is a better substrate than condensed DNA for both enzymes. This is consistent with the results of electric-dichroism studies *in vitro* with isolated chromatin [37] and of polarized fluorescence recovery after photobleaching experiments with intact nuclei

Table 1. Effect of 96 h treatment with 1 mM-DFMO or 10 μ M-BE-4-4-4 on the cell-cycle progression of U-87 MG cells

Cell-cycle distribution was measured by flow-cytometric analysis of 1×10^6 cells stained with PI, and data were analysed by the SFIT method. All data, except those for BE-4-4-4, are means $\pm$ S.D. of three to six determinations; because there was a low yield of BE-4-4-4-treated cells, the data shown are results of separate experiments.

Treatment	Cells in phase (%)		
	G1	S	G ₂ /M
Control	74.0 $\pm$ 0.7	19.0 $\pm$ 0.9	7.0 $\pm$ 0.2
DFMO	89.4 $\pm$ 0.6	6.8 $\pm$ 1.0	3.9 $\pm$ 0.4
BE-4-4-4	86.1, 86.9	7.1, 5.6	6.8, 7.6

Fig. 3. Electrophoretogram of DNA isolated from MNase-digested nuclei

Bands of nucleosomal DNA purified by electrophoresis after a 6 min digestion of nuclei with MNase on a horizontal 1.5% agarose gel. Lanes: 1 *HincII* digest of ϕ X-174 DNA (marker); 2, nuclei from control cells; 3, nuclei from DFMO-treated cells; 4, nuclei from cells treated with BE-4-4-4. Numbers in left margin denote the molecular size of marker DNA in base-pairs.

[38]. Both investigations show that multivalent cations, including spermidine and spermine, catalyse condensation of DNA in chromatin. The viscoelasticity of X-irradiated DNA isolated from DFMO-treated cells had previously suggested that depletion of polyamines causes the isolated DNA to yield smaller packets of supercoils than with DNA from untreated cells [39]. DFMO treatment, however, may have caused increased fragility of the DNA, possibly affecting its viscoelastic behaviour.

There may be several other reasons for the enhancement of nuclease-digestion kinetics of polyamine-depleted cell nuclei. The sensitivity of chromatin to MNase and DNAase I is dependent on its degree of compaction and on its transcriptional activity [19,21,40]. This sensitivity may therefore be related to cell cycle. Treatment with DFMO or BE-4-4-4 for 96 h caused a 12–15% increase in the number of cells in the G1 phase and a concomitant decrease in S-phase cells (Table 1). BE-4-4-4 had no significant effect on the number of G2/M-phase cells, whereas DFMO caused a 3–4% decrease in the number of these cells. Despite the comparable G1–S blocks induced by the two drugs, the magnitude of limit digest in BE-4-4-4-treated cell nuclei was

much higher than in those treated with DFMO (Figs. 1 and 2). Moreover, the arrest of cell-cycle progression by serum deprivation [41] caused only a small increase in the initial velocity of digestion by either enzyme and decreased the magnitude of the limit digest for both (Fig. 1). This contrasts with the increases in the extent of limit digest caused by BE-4-4-4 treatment. Therefore, the increase in the initial rates of digestion by nucleases cannot be explained by the drug-induced blockage in cell cycle.

MNase specifically digests the linker regions of chromatin. It is possible that the enhancement of the rate and extent of digestion by MNase results from a decrease in the length of nucleosomal DNA and a consequent increase in the length of linker regions. However, gel electrophoresis after a 6 min digestion with MNase resulted in very similar migration patterns for mono-, di-, tri- and oligo-nucleosomal DNA bands obtained from control cells, DFMO-treated cells and cells treated with BE-4-4-4 (Fig. 3). Therefore, neither DFMO nor BE-4-4-4 has an observable effect either on the length of the nucleosomal DNA or on that of the linker regions.

Some histone acetylases may use polyamines as substrates [42,43]. Polyamine depletion could then result in a greater degree of histone acetylation, which in turn could weaken the association between histones and DNA and increase the accessibility of DNA to nucleases. Our preliminary attempts have not shown any appreciable differences in the acetylation of core histones in control and drug-treated cells (results not shown).

In certain polynucleotides, polyamines induce B–Z transitions, which may decrease the sensitivity of chromatin to DNAase I [8,28]. Furthermore, spermine shuts off the DNAase I digestion of poly(dG-me⁵dC) by inducing a B–Z transition in the polynucleotide (results not shown), but has little effect on the digestion of calf thymus DNA. Thus the presence of less Z-like structure in cellular DNA from polyamine-depleted cells may increase the sensitivity of this DNA to the nucleases. An alteration in the degree of DNA methylation in drug-treated cells may also affect such a structural transition. However, the presence of neither B- nor Z-form DNA has been definitively shown *in vivo*.

The crystal structure of a complex of DNAase I and DNA shows that the enzyme binds to the minor groove of DNA and that this binding is sensitive to the width of the minor groove [44]. Computer graphics and solution-phase experiments provide evidence that spermine causes specific DNA sequences to bend, widening the minor groove [9–13]. This may decrease the sensitivity of DNA to digestion by DNAase I. No evidence of such polyamine-induced bending of DNA structure *in vivo*, however, has been reported so far, and no detectable levels of polyamines were found in any of the nuclei.

DNAase I has been used as a probe for transcriptionally active genes [21]. The enhanced digestion rate in polyamine-depleted cells may therefore be a result of an increase in gene transcription. The depletion of polyamines has been reported to cause selective decreases in the transcription of growth-related genes such as *c-myc*, *c-fos* and ornithine decarboxylase in human colon carcinoma cells [45]. As yet, there have been no reports of gene activation in polyamine-depleted cells. The effect of DFMO or BE-4-4-4 on the transcriptional activity of U-87 MG cells is unknown.

Polyamine depletion may alter the size of nuclear pores, which would change the nuclear permeability of nucleases. The observed enhancement of digestion could result from an enhanced uptake of the enzymes by nuclei of polyamine-depleted cells. To evaluate this possibility, we attached fluorescein isothiocyanate to the amino groups of lysine residues of DNAase I to obtain fluorescence-labelled enzyme. Conditions of labelling were carefully controlled to incorporate five fluorescein isothiocyanate molecules per molecule of enzyme. Under those conditions, no change

in the enzymic activity of DNAase I was observed in our spectrophotometric assay of digestion of calf-thymus DNA. Flow-cytometric analysis of nuclei treated with fluorescence-labelled DNAase I showed no difference in the uptake of the enzyme by the nuclei of control and of BE-4-4-4-treated cells during the first 15 min of incubation (results not shown). As enhancement of digestibility was observed during this period of incubation (Fig. 1), we conclude that the enhancement is not a result of increased uptake of the enzymes by the nuclei of polyamine-depleted cells.

Whatever the mechanism by which polyamine depletion enhances sensitivity to nucleases, it appears that the state and structure of chromatin are altered by polyamine depletion in U-87 MG cells. This conclusion corroborates that of Snyder [46], who observed that the Mg^{2+} -soluble fraction of HeLa-cell nuclei digested with either MNase or DNAase II is higher when the cells have been treated with DFMO. The extent of enhancement, however, was much smaller than the effect of BE-4-4-4 reported here. BE-4-4-4 depletes spermine, whereas DFMO does not. It is possible that the more pronounced effect on enzyme-digestion kinetics in BE-4-4-4-treated cell nuclei than in DFMO-treated cell nuclei occurs because spermine depletion is more important than the depletion of other polyamines in destabilizing chromatin structure. The intracellular presence of BE-4-4-4 may also impair chromatin condensation. BE-4-4-4 has higher affinity for DNA than spermine has, but it is poorer than spermine at condensing and aggregating DNA *in vitro* [14]. Therefore, BE-4-4-4 may displace polyamines from their binding sites on DNA, impairing chromatin condensation. Either or both of these reasons might explain the greater effect of BE-4-4-4 than of DFMO on chromatin structure. This greater effect on chromatin structure may also be a reason why BE-4-4-4 has greater growth-inhibitory and cytotoxic activity than DFMO [15].

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