Treatment with a Polyamine Analog Alters DNA–Matrix Association in HeLa Cell Nuclei: A Nucleoid Halo Assay†

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ABSTRACT: The polyamine analog 1,14-bis(ethylamino)-5,10-diazatetradecane (BE-4-4-4) depletes polyamines and inhibits the growth of tumor cells in tissue culture. We treated HeLa cells in culture with BE-4-4-4 for different time periods to produce different degrees of polyamine depletion. The cells were lysed and dehistonized to obtain nucleoids containing DNA attached to the nuclear matrix. Titration of the nucleoids with propidium iodide caused an uncoiling of negatively supercoiled DNA, resulting in the formation of a halo surrounding the nucleoid periphery. The halo diameters in both the BE-4-4-4-treated cells and the untreated control cells were measured using a fluorescence image analysis system. As compared to the control cells, the BE-4-4-4-treated cells showed a 20–25% decrease in halo diameter, indicating that there was less relaxation of the negative supercoils in the nuclear DNA of the BE-4-4-4-treated cells than in the controls.

The polyamines putrescine, spermidine, and spermine are aliphatic cations present in all mammalian cells. A depletion of intracellular polyamine levels inhibits cellular growth (Tabor & Tabor, 1984; Pegg, 1988). Most of the agents that deplete these three cellular polyamines are polyamine analogs [for a review, see Feuerstein et al. (1991), Pegg (1988), and Porter and Bergeron (1988)], some of which are ineffective as inhibitors of cellular growth (Basu et al., 1989). It is generally assumed that the only polyamine analogs that can inhibit cellular growth are those incapable of performing one or more of the intracellular functions of polyamines that relate to cell division (Basu et al., 1989; Feuerstein et al., 1991).

On the basis of our data on the effects of polyamine analogs on DNA structure and cell growth, we proposed that, in vitro, a polyamine analog that depletes cellular polyamines acts as a growth-inhibitory agent only if it fails to condense and aggregate DNA at concentrations similar to those required for spermine to condense and aggregate DNA [Basu et al., 1989, 1990a, 1992; for a review, see Feuerstein et al. (1991)]. We were encouraged in this hypothesis when 1,14-bis-(ethylamino)-5,10-diazatetradecane (BE-4-4-4),[1] a polyamine analog that aggregates DNA poorly, showed growth-inhibitory effects on several tumor cell lines (Bergeron et al., 1989; Basu et al., 1990a, 1991). Because of the inverse correlation between DNA aggregation and cytotoxicity of the analog, we hypothesized that cell division may be impaired because of the analog's inability to induce chromatin condensation or other structural transitions in specific DNA sequences. This hypothesis was supported by our finding that DNA in nuclei isolated from BE-4-4-4-treated cells exhibited increased sensitivity to micrococcal nuclease (MNase) and bovine pancreatic DNase I digestion, indicating impaired chromatin condensation and a probable loss of nucleosomes in the treated cells (Basu et al., 1992).

The formation of nucleosomes requires a negative supercoiling of DNA around core histones. In order to introduce and stabilize such supercoiling in the linear genomic DNA of eukaryotic cells, DNA needs to anchor itself to an immobilized support. Although the presence of a protein-rich nuclear fraction after extraction of the nuclear membrane and chromosomes was reported in the early 1960s (Zbarsky et al., 1962), the importance of this structure, the "nuclear matrix", was not realized until it was found that eukaryotic DNA replication takes place at specific sites of DNA attachment to this structure and that the periodic attachment of specific DNA sequences to the nuclear matrix has a pivotal role in gene expression and replication (Pardoll et al., 1980; Nelkin et al., 1982; Razin, 1987; Dijkwel & Hamlin, 1988; Ip et al., 1988; Tsuitsui et al., 1988; Hirose & Ohta, 1990; Vaughn et al., 1990; He et al., 1991). Little is known, however, about the cellular components that mediate and stabilize the attachment of DNA sequences to matrix at particular sites. Such mediation is a key factor in the regulation of important biologic processes such as transcription and replication which control cell division and proliferation.

A simple method has been developed for studying the changes in DNA–matrix attachment and the state of supercoiling of DNA in nuclei by using propidium iodide titration of dehistonized nucleoids (Roti-Roti & Wright, 1987; Wright et al., 1990). Propidium iodide causes uncoiling of the negatively supercoiled DNA, resulting in the formation of a fluorescent halo of propidium-bound DNA surrounding the periphery of the nuclear matrix. The diameter of this halo can be measured using a fluorescence image analysis system, and changes in the diameter of the halo can be related to the extent of DNA–matrix attachment and the degree of DNA supercoiling in the nuclei. It may be presumed that polycations like spermine or spermidine should stabilize complexes between

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[1] Abbreviations: BE-4-4-4, 1,14-bis(ethylamino)-5,10-diazatetradecane; MNase, micrococcal nuclease; MEM, minimum essential medium; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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negatively charged DNA and the nuclear matrix, which consists of large quantities of polyanions such as acidic proteins and RNA (Narayan et al., 1967). However, to the best of our knowledge there are no reports on the role of polyamines or their analogs in DNA–matrix attachment and associated cellular processes.

We report the effect of polyamine depletion induced by the polyamine analog BE-4-4-4 on the DNA halo of HeLa cell nucleoids stained with propidium iodide at various stages of polyamine depletion. As the period of incubation increased, BE-4-4-4 inhibited cell growth and caused both a gradual decrease in polyamine levels and a markedly smaller nucleoid halo diameter than was seen in untreated cells.

MATERIALS AND METHODS

Materials

BE-4-4-4 was the generous gift of Prof. Raymond J. Bergeron of the University of Florida, Gainesville, FL. Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade, and double distilled deionized water was used as solvent.

Methods

Tissue Culture. HeLa cells (5×10^4/mL) were seeded into spinner bottles in 100 mL of Joklik's modified MEM supplemented with 3.5% fetal bovine and 3.5% newborn calf serum (Roti-Roti & Wright, 1987). The flasks were incubated and stirred slowly in a 37 °C chamber. Cells were grown for at least 24 h to ensure that they were in the log phase of growth before they were treated with BE-4-4-4. A stock solution of 10 mM BE-4-4-4 was prepared in Hanks' balanced salt solution with the pH adjusted to 7.2 and was sterile-filtered immediately before use (Basu et al., 1990b). Cells were harvested in triplicate at different time points after treatment and were counted using an electronic particle counter. Untreated cells served as control.

Polyamine Measurement. Approximately (5–10) $\times 10^5$ cells were collected from each sample and were washed twice with isotonic phosphate buffer (pH 7.4). Cells were sonicated in 250 μL of 8% sulfosalicyclic acid and then centrifuged, and 50–100 μL of the supernatant was dansylated and analyzed for polyamine content using high-performance liquid chromatography (Kabra et al., 1986).

Nucleoid Halo Assay. To visualize and measure the DNA loops in the nucleoid halo, cells were resuspended to a concentration of (1–2) $\times 10^5$/mL in Earle's balanced salt solution (Kampinga et al., 1988). An aliquot of cell suspension (250 μL) was placed in each well of a four-well LabTek slide (Miles Scientific, Naperville, IL). Cells were incubated in the dark with an equal volume of lysis buffer [2.0 M NaCl, 20 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 0.5% Triton X-100] containing twice the desired concentration of propidium iodide for 10–15 min. The images were visualized by using an SIT TV camera and monitor with a Model 3000 image analyzer (Image Technology Corp., New York, NY) connected to an IBM PC-based analysis system. The diameter of a nucleoid halo was determined across several directions, and the mean diameter and standard deviation were calculated. One-dimensional gel electrophoresis of proteins isolated from the nuclear matrix of control HeLa cells and cells treated with 10 μM BE-4-4-4 was also performed to assess changes in the protein compositions of the nuclear matrix.

Histone Displacement Study. Cells [(1.5–2) $\times 10^6$] were lysed in 5-mL nitrocellulose ultracentrifuge tubes by mixing equal volumes of cell suspension and lysis solution [2 M NaCl, 20 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 0.5% TX-100] containing the appropriate concentration of propidium iodide. After 30 min, nucleoids were recovered from the cell lysates by centrifugation at 150000*g* for 30 min at 4 °C. Supernatants were carefully decanted, tube walls were dried, and the pellets were resuspended in 25 μL of digestion buffer [10 mM Tris (pH 7.4), 5 mM $MgCl_2$, 10 mM NaCl, and 0.1 mM freshly added PMSF]. DNase (chromatographically purified; Worthington Biochemical Corp., Freehold, NJ) and RNase (preboiled for 5 min; Sigma Chemical Co.) were added to give final concentrations of 0.625 and 0.125 mg/mL, respectively. After digestion at room temperature for 5 min, an equal volume of 2 × SDS sample buffer [4% SDS, 120 mM Tris-HCl (pH 6.8), 20% glycerol, 200 mM DTT, and 0.1% bromophenol blue] was added, and the samples were placed in boiling water for 5 min. The protein samples were stored at −80 °C until analysis by using one-dimensional polyacrylamide gel electrophoresis in the presence of SDS following conditions described by Laemmli (1970). After electrophoresis, protein bands were scanned and quantitated using a densitometer (Molecular Dynamics, Sunnyvale, CA) equipped with image quantitating softwares.

RESULTS AND DISCUSSION

Within 24 h after treatment with the polyamine analog BE-4-4-4 (10 μM), HeLa cells were completely depleted of putrescine, and their spermidine and spermine levels were reduced to ≈10% and ≈50% of control levels, respectively. Both the spermidine and spermine levels decreased further with longer periods of incubation (Figure 1). Within 48 h after treatment, inhibition of cell growth was apparent, and after 72 h, the number of BE-4-4-4-treated cells was half the number of untreated control cells (Figure 1).

The diameters of the halos of the untreated cells and of cells treated with BE-4-4-4 for different time periods were plotted against propidium concentrations. These data are shown separately (Figure 2A,B) because the experiments were performed in two different sets, and the control titration profiles, although similar in magnitude, showed some difference in shape that may be related to growth status, incubation conditions, and/or other uncontrolled environmental conditions that may have varied between the two sets. The halo diameter increased with an increase in the propidium concentration as more and more negative supercoils in DNA unfolded and eventually reached a maximum diameter at a propidium concentration of 7.5 μg/mL. Thereafter, further increases in the propidium concentration introduced positive supercoiling and consequently a decrease in the halo diameter. At a high propidium concentration (50 μg/mL), the supercoils wound back to form halos of similar diameters in the BE-4-4-4-treated and untreated cells, suggesting that neither the nucleoid preparation procedure nor exposure to BE-4-4-4 induces DNA strand breakage.

The maximum halo diameter in the cells treated with BE-4-4-4 for 72 h was significantly smaller than that in the control cells (Figure 2A). The halo diameter decreased with an increase in the duration of BE-4-4-4 treatment from 24 to 48 h, and then plateaued (Figure 2A,B). After 72 h of treatment, the maximum halo diameter of nucleoids from the analog-treated cells was about 20–25% smaller than that of nucleoids from control cells (Figure 2A). This degree of reduction in

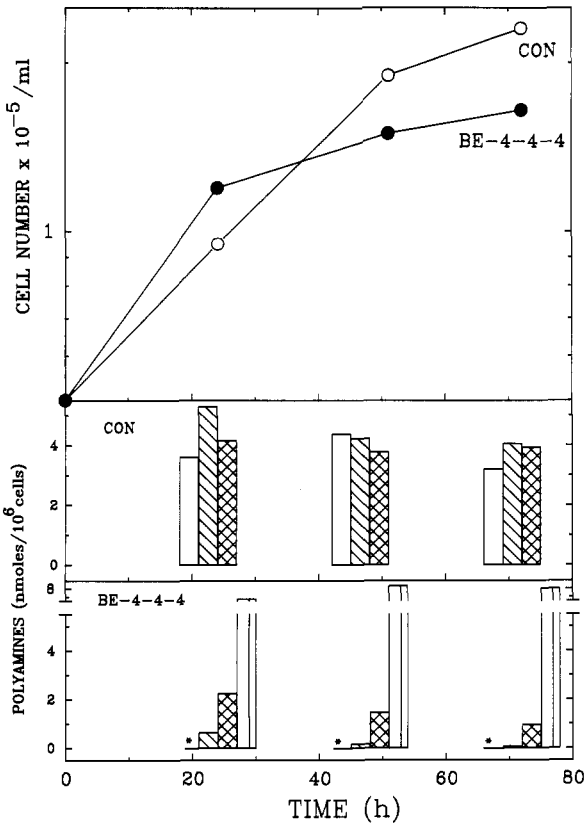

FIGURE 1: Effect of 10 μM BE-4-4-4 on the growth (panel A) and polyamine levels (panel B) of HeLa cells. Each point in the growth curve is an average of three independent experiments. Error bars are smaller than the symbol size. Control (open circles); BE-4-4-4 (closed circles). Each point in polyamine level is an average of two separate measurements. Putrescine (open bars); spermidine (hatched bars); spermine (cross-hatched bars); BE-4-4-4 (divided bar). The asterisks denote polyamines undetectable with high-performance liquid chromatography.

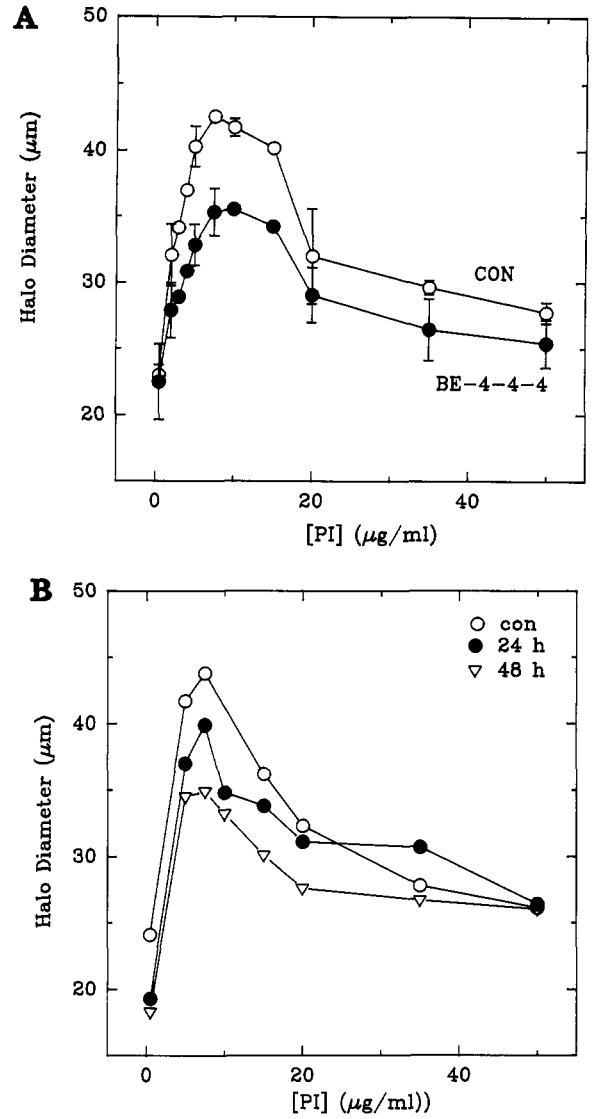

FIGURE 2: (A) Diameters of nucleoid halos of HeLa cells treated with 10 μM BE-4-4-4 for 72 h. Control (open circles); BE-4-4-4 (closed circles). Points and standard deviations were calculated on the basis of data from three separate experiments. In each experiment, diameters of at least 40 nucleoids were measured, and mean diameters were calculated using a computer-based image analysis system. (B) Diameters of nucleoid halos of HeLa cells treated with 10 μM BE-4-4-4 for different time periods. Control (open circles); 24-h treatment (closed circles); 48-h treatment (open triangles). Each point was calculated on the basis of data from at least two separate experiments. In each experiment, diameters of at least 40 nucleoids were measured, and mean diameters were calculated using a computer-based image analysis system.

the nucleoid halo diameter is the greatest that has been evident for any agent tested so far (J. Roti-Roti, unpublished data).

As reduction in the halo diameters preceded growth inhibition by 24 h, it appears that a BE-4-4-4-induced process(es) leading to a decrease in the halo diameter is (are) likely to be the cause, and not an effect, of the growth inhibition. Both the intracellular level of polyamine analog and the decrease in the halo diameter plateaued by 24–48 h of treatment (Figure 1). The cellular polyamine levels, however, progressively decreased for 72 h. Although it could be that reduction in intracellular polyamine concentrations to a point below a certain level affects the halo diameter, it seems more likely that the intracellular presence of BE-4-4-4, rather than the depletion of cellular polyamines, is responsible for the decrease in halo diameters and ultimately for cell death. This may be supported by the fact that BE-4-4-4 can inhibit the growth of genetically engineered Chinese hamster ovary cells even though it does not completely deplete cellular polyamines (Ghoda et al., 1992). At this point, however, we cannot exclude the possibility that the effect of BE-4-4-4 on the halo diameter may not be related, at least in part, to its ability to decrease cellular polyamine levels.

The most likely reason for the decrease in the halo diameter in treated cells seems to be a decrease in the number of nucleosomes as a result of a failure of BE-4-4-4 to properly catalyze the process of chromatin condensation. We have previously shown that treatment with BE-4-4-4 enhances the MNase and DNase I sensitivity of nuclei of U-87 MG human brain tumor cells (Basu et al., 1992). Our [^{3}H]thymidine incorporation studies also have shown that newly synthesized DNA is incorporated into nucleosomes of U-87 MG cells in smaller amounts after BE-4-4-4 treatment (Basu et al., unpublished results). Either an unmasking of existing matrix attachment regions in DNA due to the loss of nucleosomes or a stabilization of anchor points that are usually disrupted during normal isolation procedures, or a combination of both, seems to be the most likely reason for the decrease in the apparent DNA loop size and consequently a decrease in the halo diameter. Although the appearance of new matrix attachment sites in BE-4-4-4-treated cells cannot be completely ruled out, the possibility of the loss of nucleosomes was substantiated by the histone displacement assay of the nucleoids (Table I). Significantly lesser amounts of H2A,

Table I: Core Histones Remaining in Propidium Iodide-Treated Nucleoids

propidium iodide concn (μg/mL)	control (density units)	BE-4-4-4[a] (density units)
0	1989.7	911.0
0.5	1854.1	840.7
2.0	1296.9	607.3
5.0	810.6	402.3
7.5	626.2	126.0
50.0	164.6	77.4

[a] 10 μM for 72 h.

H2B, H3, and H4 histones are found associated with nucleoids from BE-4-4-4-treated cells as compared to nucleoids from control untreated cells at all concentrations of propidium iodide used.

The exact reason for the decrease in the halo diameter of polyamine analog-treated HeLa cells is still unknown. It is possible that BE-4-4-4, which aggregates DNA poorly, impairs chromatin condensation and inhibits nucleosome formation, decreasing the degree to which negative supercoils in chromosomal DNA relax during propidium iodide titration.

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