Differential Effect of Ethidium Bromide and Cytosine Arabinoside on Mitochondrial and Nuclear DNA Synthesis in HeLa Cells

KENZO KATO *, KLAUS D. RADSAK **, and HILARY KOPROWSKI

The Wistar Institute of Anatomy and Biology, 36th and Spruce Streets, Philadelphia, Pennsylvania 19104

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The effect of ethidium bromide (EB) on the synthesis of circular DNA of mammalian cells was studied by isopycnic centrifugation in a CsCl-EB solution. EB (0.1 – 0.5 µg/ml) interferes with the synthesis of newly-formed circular DNA of HeLa cell mitochondria and causes degradation of the pre-existing circular DNA, as well. Under the same conditions, nuclear DNA synthesis was not inhibited. This effect was not reversible at a concentration of 0.5 µg EB/ml or more. Cytosine arabinoside (ara-C) did not exhibit an effect similar to that of EB.

Recently we have observed that ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide) (EB) at concentrations of 0.1 µg/ml or more inhibits the incorporation of 3H-thymidine into the circular double-stranded DNA of mitochondria in HeLa cells. The question arose whether EB only inhibits the synthesis of the newly-formed circular DNA of mitochondria or also degrades pre-existing circular DNA. The reversibility of the EB effect was another point to be clarified. Of importance also was a comparison of the effect of EB with that of cytosine-arabinoside (ara-C).

Materials and Methods

Cells: M HeLa cells were grown as monolayer cultures in double-strength Eagle’s Basal Medium (2X BME) supplemented with 10% fetal calf serum (FCS).

Chemicals: EB was purchased from Calbiochem, Los Angeles, Calif., 3H-thymidine (specific radioactivity, 20 Ci/m mole) and 14C-thymidine (specific radioactivity, 24.5 mC/m mole), from New England Nuclear, Boston, Mass., and cytosine-arabinoside from Sigma Chemical Company, Missouri.

Extraction of mitochondrial DNA, centrifugation conditions, and measurement of radioactivity: Crude preparations of mitochondrial DNA of HeLa cells were obtained by the extraction method using 1% sodium dodecyl sulfate (SDS) and 100 µg predigested pronase per ml and by the fractionation procedure described by Hirt. Isopycnic centrifugation of the supernatant was performed in a CsCl solution containing 100 µg EB/ml (mean density, 1.550) for 24 h at 43,000 rev/min at 20°C (see ref. 2) using at 50 fixed-angle rotor in a Beckman Model L2 centrifuge. Nuclear DNA was extracted as described previously.

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The fractions were collected from the bottom of the gradients and precipitated on millipore filters (0.45 µ) with cold trichloracetic acid as described previously. The radioactivity was measured in a Beckman liquid scintillation counter using a toluene — Liquiflour (New England Nuclear) mixture.

Results

Effect of EB on the circular DNA of HeLa cell mitochondria: Previous experiments showed that EB inhibits the synthesis of newly-formed circular mitochondrial DNA in HeLa cells. To examine whether it also acts on pre-existing circular DNA, we labeled approximately 5 × 10^6 HeLa cells with 14C-thymidine (1.0 µC/ml) for 48 h. The medium was then changed, and the cells were exposed to 3H-thymidine (5 µC/ml) and EB (0.5 µg/ml) for an additional 48 h. No EB was added to the control culture. After a total incubation period of 96 h, the mitochondrial DNA was extracted by the SDS-pronase method described by Hirt. If EB interfered only with the synthesis of newly-formed circular DNA, 14C-labeled, covalently-closed, circular, double-stranded DNA would be detectable by isopycnic centrifugation in a CsCl-EB solution. If, however, EB also degraded pre-existing circular DNA, no radioactivity should occur in the position of double-stranded circular DNA.

In the control samples, a clear radioactivity peak was obtained for 3H and 14C in the region of the circular mitochondrial DNA (Fig. 1 a), whereas in the EB-treated samples no specific radioactivity peak was exhibited in this region (Fig. 1 b).

The total 3H-radioactivity of circular DNA region in the EB-treated sample was 3.2% of that in the

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Treatment of cells Total radioactivity ($^{14}\text{C}$) of circular DNA fraction

<table>
<thead>
<tr>
<th>Treatment of cells [µg EB/ml for 48 h]</th>
<th>0.5</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>net counts/min  % control</td>
<td>26</td>
<td>316</td>
</tr>
<tr>
<td>8.2% controls</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Degradation of Pre-Existing Closed-Circular Mitochondrial DNA in HeLa Cells by Ethidium Bromide.

Table 3. Reversibility of the Effect of Ethidium Bromide.

Reversibility of the effect of EB: To examine whether the effect of EB is reversible, we exposed $5 \times 10^6$ HeLa cells to concentrations of 0.1 or 0.5 µg EB/ml of culture medium for 48 h. The medium was then changed and the cells were continuously labeled with $^3\text{H}$-thymidine (5 µC/ml) for an additional 48 h. The mitochondrial DNA was then extracted by the SDS-pronase method of HIRT and analyzed by isopycnic centrifugation in a CsCl-EB solution.

Limited radioactivity was obtained in the position of circular double-stranded DNA. In this case the total radioactivity for the circular DNA region in the EB-treated samples was 22.4% (0.1 µg EB/ml) and 9.6% (0.5 µg EB/ml) of the control (Table 3).

Comparison of the effect of EB with that of cytosine-arabinoside: To ascertain the specificity of the action of EB, the effect of cytosine-arabinoside was examined in the same system. The compound was added simultaneously with $^3\text{H}$-thymidine (5 µC/ml) to the culture medium of approximately $5 \times 10^6$ HeLa cell monolayer cultures. After 48 h, the mitochondrial DNA was extracted and analyzed by isopycnic centrifugation in a CsCl-EB solution.

As shown in Fig. 2, at the chosen concentrations cytosine-arabinoside (10 µg/ml) did not exhibit an effect similar to that of EB. In contrast to EB, which
Fig. 2. Effect of EB and cytosine-arabinoside on the synthesis of mitochondrial DNA in HeLa cells. Isopycnic centrifugation in an CsCl-EB solution was performed on mitochondrial DNA, (a) extracted from $5 \times 10^8$ HeLa cells labeled with $\alpha$-thymidine ($5 \mu$C/ml) for 48 h; (b) extracted from $5 \times 10^8$ HeLa cells labeled with $\alpha$-thymidine for 48 h and simultaneously exposed to 0.5 $\mu$g EB/ml; (c) extracted from $5 \times 10^8$ HeLa cells labeled with $\alpha$-thymidine ($5 \mu$C/ml) for 48 h and simultaneously exposed to cytosine-arabinoside ($10 \mu$g/ml). Centrifugation conditions: CsCl-EB (100 $\mu$g/ml); mean density 1.55; 50 fixed-angle rotor; 43,000 rev/min; 24 h; 20°C. The buoyant densities ($\bullet—•$) of circular DNA are 1.607 (a) and 1.606 (b), of linear DNA 1.564 (a), 1.570 (b), 1.568 (c). The linear DNA fraction represents contaminating nuclear DNA fragments (see legend Fig. 1).

strongly inhibits the synthesis of mitochondrial DNA, but does not affect that of nuclear DNA, cytosine-arabinoside strongly inhibited the nuclear DNA synthesis (Table 4) and slightly the mitochondrial DNA synthesis (Table 4) and slightly the mitochondrial DNA synthesis (Fig. 2 c). In Fig. 2 c, most of the radioactivity in the region of linear DNA ($\varphi = 1.568$) may be explained by the observation that cytosine-arabinoside causes a large amount of degradation of nuclear DNA (unpublished data).

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>Specific radioactivity of nuclear DNA as % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide</td>
<td>1.0 94</td>
</tr>
<tr>
<td></td>
<td>0.5 116</td>
</tr>
<tr>
<td>Cytosine-arabinoside</td>
<td>10 1</td>
</tr>
<tr>
<td>Control</td>
<td>1 14</td>
</tr>
</tbody>
</table>

Table 4. Comparison of the Effects of EB and Cytosine-Arabinoside on the Synthesis of Nuclear DNA in HeLa Cells.

* Mean value, 2196 counts/min $\alpha$H/$\mu$g DNA.

Discussion

Our results show that EB interferes differentially with the synthesis of closed circular double-stranded DNA of mitochondria in HeLa cells. It also degrades preexisting circular DNA. These effects are not reversible during the time period observed.

The action of EB, therefore, cannot be explained by the specific inhibition of the mitochondrial DNA polymerase alone. Its effect may also be the consequence of a degradation of the circular DNA, caused by direct interaction between DNA and EB. The degradation might furthermore result from an indirect action of EB on the circular DNA by the inactivation of repair enzymes or activation of endonucleases. Further experiments are needed to elucidate this result.

In any case differential inhibition of the synthesis of circular DNA of mitochondria with EB could be a useful tool in studying the as-yet-unknown relation between nuclear- and mitochondrial-DNA-directed apparatus of the cell. Investigations in this direction are in progress.

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