Excretion of Spermidine from BHK-21/C13 Cells Exposed to 6-Thioguanosine

Maureen A. L. Melvin, William T. Melvin, and Hamish M. Keir

Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, Scotland, United Kingdom

ABSTRACT

The growth of BHK-21/C13 cells in monolayer cultures was inhibited by 6-thioguanosine. Accumulation of putrescine, spermidine, and spermine was inhibited by 6-thioguanosine, and cells incubated in the presence of the drug had a decreased content of polyamines relative to control cells. These effects were more marked for spermidine than for spermine or putrescine. Consequently, the intracellular spermidine:spermine molar ratio was decreased in cells exposed to the drug. Cells, the polyamines of which had been labeled with [3H]putrescine, were incubated in the presence or absence of 6-thioguanosine. More polyamines were lost from cells exposed to the drug than from control cells. The radioactive material excreted was predominantly spermidine, both as its free form and in a conjugated form, even when the cells contained large amounts of labeled spermine. This release of spermidine from BHK-21/C13 cells into the culture medium was a specific response of the cells to alterations in growth potential rather than a consequence of leakage due to cell lysis.

INTRODUCTION

Polyamine levels are elevated in the urine and serum of patients with cancer (1, 18), and increased urinary polyamine levels have been observed after successful treatment of various types of cancer (18). Consequently, it has been suggested that levels of polyamines, particularly spermidine, in physiological fluids may be useful to clinicians in assessing tumor response to chemotherapy (5, 18). Dramatic increases in the levels of extracellular spermidine in tumor-bearing animals occurred in response to chemotherapy (21) and to tumor regression obtained by hormonal deprivation (19). Following local X-irradiation the spermidine content of rat hepatoma tumors decreased while spermidine levels in the serum increased; the concentration of polyamines in the liver remained unchanged (20). These results suggested that intracellular spermidine, which increases during tumor growth, is diminished by excretion during regression and that spermidine concentrations in extracellular fluids may reflect tumor cell death.

We have shown previously that inhibition of the growth rate of BHK-21/C13 cells in culture by depriving the cells of serum resulted in depletion of the intracellular polyamine content and release of polyamines, particularly spermidine, from the cells into the culture medium (12, 13). The present study was undertaken to examine the effects of 6-TGR on the growth of BHK-21/C13 cells and on the fate of intracellular polyamines. 6-TGR is incorporated into cellular nucleic acids after phosphorylation (16). It may first be converted to 6-thioguanine, which is an agent clinically useful in the treatment of acute leukemia (4, 6).

MATERIALS AND METHODS

Materials. [1,4(14)H]Putrescine dihydrochloride (19 Ci/mmol), [6-3H]thymidine (21.5 Ci/mmol), [5-3H]uridine (28.1 Ci/mmol), and L-[4,5-3H]leucine (40 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Buckinghamshire, United Kingdom. Nonradioactive hydrochlorides of putrescine, spermidine, and spermine; 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride; and 6-mercaptopurine (6-TG) were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, United Kingdom. L-Proline was from BDH Biochemicals Ltd., Poole, Dorset, United Kingdom. Materials for tissue culture, including horse serum screened for Mycoplasma, were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, and Silica Gel 60 thin-layer chromatography plates (20 x 20 cm) were from Merck AG, Darmstadt, West Germany.

Cell Culture. BHK-21/C13 cells (11) were grown in monolayer cultures at 37° in a 5% CO2 atmosphere in 2.24-liter roller bottles in ETH10 medium until about 2 generations short of confluence. The cell sheet was then washed with buffered trypsin solution, which was immediately poured off, and the cells were left at room temperature until they detached from the glass. The cells were washed off the glass with ETH10 medium, and after cell number was determined with an improved Neubauer hemocytometer they were dispersed into 90-mm-diameter vented plastic dishes. These were incubated at 37° in a 5% CO2 atmosphere for 24 hr to allow the cells to attach to the plastic and commence growth. The medium was then replaced with fresh ETH10 medium containing various concentrations of 6-TG, which had been dissolved in Eagle’s medium immediately before use and sterilized by filtration. Cells were checked routinely for contamination by Mycoplasma.

Estimation of Cell Growth. Protein, RNA, and DNA were extracted from cells and estimated quantitatively as described previously (13, 14). Putrescine, spermidine, and spermine, extracted from cells by ice-cold 0.2 M perchloric acid, were converted to their dansylated derivatives and separated by thin-layer chromatography, the chromatography plates (20 x 20 cm) were from Merck AG, Darmstadt, West Germany.
grams being developed twice in ethyl acetate:cyclohexane (2:3, v/v). The amines were determined quantitatively by measurement of the fluorescence of their dansylated derivatives (9).

**Synthesis of DNA, RNA, and Protein.** Cells were dispensed into 90-mm-diameter dishes at a density of 1.0 × 10⁶ cells/dish and were incubated overnight as described above before being transferred to fresh medium containing 6-TGR. At the appropriate time thereafter, the medium was replaced by fresh medium with or without 6-TGR. At the appropriate time thereafter, the medium was replaced by fresh medium containing [6-3H]thymidine (0.5 μCi/ml), [5-3H]uridine (5.0 μCi/ml), or L-[4,5-3H]leucine (2.0 μCi/ml). The radioactive medium was left in contact with the cells for 1 hr at 37° in a 5% CO₂ atmosphere. The cells were then removed, and the cells were washed with ice-cold PBS and scraped off the dishes with ice-cold 0.2 M perchloric acid. The precipitate was collected by centrifugation at 200 × g, for 5 min, washed with 0.2 M perchloric acid, and solubilized with 0.3 M NaOH. Aliquots were neutralized with trichloroacetic acid and assayed for radioactivity by liquid scintillation spectroscopy with a Triton X-100:toluene (1:2, v/v) scintillation fluid containing 4 g of PPO and 0.05 g of POPOP per liter in an Intertechnique SL40 liquid scintillation spectrometer with approximately 40% efficiency for 3H.

**Radioactive Labeling of Intracellular Polyamines and Their Determination in Cells and Medium.** Intracellular polyamines were labeled by incubating nonconfluent cells growing in 2.24-liter roller bottles in ETH₀ medium for 20 hr in the presence of [1,4(n)-3H]putrescine dihydrochloride (1.0 μCi/ml). The radioactive medium was poured off, and the cell sheet was washed twice with ETH₀ medium before harvesting. Cells were dispensed into 90-mm-diameter vented plastic dishes at a density of 0.75 × 10⁶ cells/dish and were incubated overnight in ETH₀ medium, after which the medium was replaced by fresh medium with or without 6-TGR. After incubation for the required time at 37°, the medium was decanted and centrifuged at 200 × g, for 5 min to remove cell debris. Portions of the medium were: (a) assayed directly for radioactivity; (b) treated with ice-cold 0.2 M perchloric acid, with the extract of polyamines danylated, separated by thin-layer chromatography, and assayed for radioactivity (13); or (c) washed with ice-cold trichloroacetic acid [final concentration, 4% (v/v)], followed by anhydrous ether, with the extract hydrolyzed with 6 M HCl at 110° for 1 hr and then dried to a powder form (7). Samples were dissolved in 0.2 M perchloric acid and treated with dansyl chloride, and the products were separated and analyzed for radioactivity.

The cell sheet was washed 3 times with ice-cold PBS to remove any labeled polyamines absorbed to the cell surface, and the cells were scraped off the dishes with ice-cold PBS. The polyamines were extracted with either (a) ice-cold perchloric acid or (b) trichloroacetic acid followed by hydrolysis with 6 M HCl, and the polyamines were dansylated and analyzed as described above.

**RESULTS**

**Effect of 6-TGR on Cell Growth.** BHK-21/C13 cells growing in 90-mm-diameter dishes were transferred to fresh medium containing various concentrations of 6-TGR. The amounts of protein, RNA, and DNA per culture were determined before transfer (the initial culture value) and 24 or 46 hr after transfer. Cell proliferation and the accumulation of macromolecules were inhibited by 6-TGR, the amount of inhibition increasing with increased concentration of 6-TGR in the medium and with increased time of exposure of the cells to the drug (Table 1). There was little cell lysis as determined by microscopic examination and permeability of the cells to trypan blue. The inhibition was not reversed when 6-TGR-treated cells were transferred to fresh medium containing no drug.

Inhibition of the synthesis of DNA, RNA, and protein was observed within a few hr of exposure of BHK-21/C13 cells to 6-TGR, the amount of inhibition increasing with increased drug concentration (Table 2). At higher concentrations of 6-TGR (10 to 100 μM), protein synthesis was affected less than was DNA and RNA synthesis after a 6-hr exposure of the cells to the drug, a result that is consistent with the data presented in Table 1.

**Effect of 6-TGR on Accumulation of Putrescine, Spermidine, and Spermine.** Within 27 hr of transfer of growing BHK-21/C13 cells to fresh medium containing 6-TGR, the accumulation of cellular polyamines was inhibited to an extent dependent on drug concentration (Table 3). Accumulation of spermidine was inhibited more than was accumulation of spermine, so that the molar ratio of spermidine to spermine decreased drug concentration (Table 2). At higher concentrations of 6-TGR (10 to 100 μM), protein synthesis was affected less than was DNA and RNA synthesis after a 6-hr exposure of the cells to the drug, a result that is consistent with the data presented in Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>6-TGR (μM)</th>
<th>Time (hr)</th>
<th>Protein (mg)</th>
<th>RNA (μg)</th>
<th>DNA (μg)</th>
<th>Cell no. (x10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24</td>
<td>2.3</td>
<td>211</td>
<td>45</td>
<td>5.5</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
<td>3.5</td>
<td>314</td>
<td>101</td>
<td>12.3</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>1.5</td>
<td>152</td>
<td>33</td>
<td>5.0</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>2.6</td>
<td>173</td>
<td>58</td>
<td>4.3</td>
</tr>
<tr>
<td>30</td>
<td>24</td>
<td>1.7</td>
<td>140</td>
<td>45</td>
<td>2.7</td>
</tr>
<tr>
<td>Initial</td>
<td>0</td>
<td>0.6</td>
<td>76</td>
<td>16</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*These results are from an experiment different from the others described in the table.*

**Table 2**

<table>
<thead>
<tr>
<th>6-TGR (μM)</th>
<th>[³H]Thymidine (%)</th>
<th>[³H]Uridine (%)</th>
<th>[³H]Leucine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>70</td>
<td>79</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>43</td>
<td>29</td>
<td>58</td>
</tr>
<tr>
<td>30</td>
<td>26</td>
<td>24</td>
<td>50</td>
</tr>
<tr>
<td>100</td>
<td>16</td>
<td>18</td>
<td>51</td>
</tr>
</tbody>
</table>

© 1978 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on April 15, 2017.
to spermine decreased with increasing drug concentration.

Cells exposed to 6-TGR generally had a lower content of polyamines than did cells incubated in the absence of drug. For example, cells incubated in the presence of 10 μM 6-TGR for 27 hr contained 6.7 nmol spermine, 11.9 nmol spermidine, and 2.2 nmol putrescine per mg protein, whereas control cells contained 7.6 nmol spermine, 16.7 nmol spermidine, and 3.2 nmol putrescine per mg protein. The difference was always more marked for spermidine than for spermine or putrescine and was reflected in the decrease in the spermidine:spermine molar ratio (Table 3).

Fate of Intracellular Polyamines. BHK-21/C13 cells, the polyamines of which had been labeled with tritium, were transferred to fresh medium containing various concentrations of 6-TGR, and the cells and the medium in which they were incubated were analyzed for radioactivity at various times thereafter (Chart 1). Cells exposed to 6-TGR lost considerably more radioactivity into the culture medium than did control cells. Maximal release of radioactivity into the medium corresponded with maximal inhibition of cell growth by the drug (Table 1).

Identification of Labeled Material in Cells and Medium. Aliquots of the cells and medium of cultures of BHK-21/C13 cells incubated for 46 hr in the presence of either no drug or 10 μM 6-TGR (Chart 1) were extracted with ice-cold 0.2 M perchloric acid, the extracts were treated with dansyl chloride, and the products were separated by thin-layer chromatography and analyzed for radioactivity ["unhydrolyzed" samples (Table 4)]. A significant proportion of the radioactivity in all samples was present in a form that migrated no further than 1.0 cm from the origin of the chromatograms. Cells incubated in the presence of 6-TGR generally contained an increased proportion of their radioactivity in this material compared with control cells. The remainder of the radioactivity inside the cells was divided between spermine and spermidine, whereas in the samples of medium the remainder of the radioactivity was located predominantly in spermidine. This indicates that loss of polyamines from the cells was not simply a consequence of cell lysis; otherwise more radioactivity would have been present in spermine in the medium.

Portions of the cells and medium were extracted with ice-cold trichloroacetic acid, and the extracted material was hydrolyzed with 6 M HCl and then dansylated and analyzed ["hydrolyzed" samples (Table 4)]. For all samples hydrolysis decreased the amount of radioactivity found at the origin of the chromatograms and increased the radioactivity located in spermidine and spermine. This indicates that this material represented conjugated polyamines. The radioactivity in the cells was again distributed between spermine and spermidine, but the radioactivity in the medium was still located predominantly in spermidine. This suggests that spermidine was excreted specifically from the cells into the culture medium (13).

DISCUSSION

It has been shown previously that inhibition of the growth rate of mammalian cells in culture by deprivation of growth factors (10, 13) or by heat shock treatment (7) resulted in depletion of intracellular polyamines, particularly spermidine, and increased loss of polyamines from the cells into the culture medium. The present study shows that when the

---

**Table 3**

**Effect of 6-TGR on the accumulation of putrescine, spermidine, and spermine**

Four dishes were used for each determination of putrescine, spermidine, and spermine. The values presented are the average contents/dish from triplicate assays. All estimates varied by less than 10% of the values shown.

<table>
<thead>
<tr>
<th>6-TGR (μM)</th>
<th>Spermine (nmol/dish)</th>
<th>Spermidine (nmol/dish)</th>
<th>Putrescine (nmol/dish)</th>
<th>Spermidine:spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.4</td>
<td>27.2</td>
<td>5.2</td>
<td>2.19</td>
</tr>
<tr>
<td>1</td>
<td>12.4</td>
<td>25.4</td>
<td>5.1</td>
<td>2.08</td>
</tr>
<tr>
<td>3</td>
<td>8.9</td>
<td>15.6</td>
<td>3.7</td>
<td>1.75</td>
</tr>
<tr>
<td>10</td>
<td>5.6</td>
<td>10.0</td>
<td>1.9</td>
<td>1.78</td>
</tr>
<tr>
<td>30</td>
<td>5.3</td>
<td>7.8</td>
<td>1.5</td>
<td>1.48</td>
</tr>
<tr>
<td>100</td>
<td>4.0</td>
<td>4.9</td>
<td>1.1</td>
<td>1.22</td>
</tr>
<tr>
<td>Initial culture</td>
<td>2.7</td>
<td>6.1</td>
<td>1.0</td>
<td>2.24</td>
</tr>
</tbody>
</table>
growth of BHK-21/C13 cells in culture was inhibited by 6-TGR the accumulation of polyamines was inhibited, the intracellular content of polyamines was decreased, and the loss of polyamines from the cells into the culture medium was considerably increased compared to control cells. The nature of the polyamines released from the cells was predominantly spermidine, which was found both free and as a "conjugated" form in the medium. Conjugation of spermidine took place intracellularly and may perhaps be a prerequisite for excretion (17). These results support the hypothesis that excretion of spermidine from BHK-21/C13 cells is a specific response to cessation of cell growth (13) and may be an important mechanism whereby these cells dispose of polyamines present in excess of their requirements. In this study loss of spermidine from the cells does not seem to be due simply to cell death and lysis (18).

This work may have implications concerning the clinical effects of 6-thiopurines and other anticancer agents, in particular their effects on the levels of polyamines in physiological fluids (5). We have shown recently that inhibition of the growth of BHK-21/C13 cells by the antitumor agent methylglyoxal bis(guanylhydrazone) resulted in diminished excretion of intracellular polyamines from the cells when compared with cells exposed to no drug. This difference probably reflects the different mechanisms of action of the drugs on the cells; 6-TGR interferes with the nucleic acid metabolism of cells (2, 3, 15, 16), whereas methylglyoxal bis(guanylhydrazone) has potent effects on the pathway of biosynthesis of the polyamines (8).

ACKNOWLEDGMENTS

We should like to acknowledge the skilled assistance of Hamish Allen, who conducted some of the preliminary experiments for this study. We are grateful to Alison Blair for her excellent technical assistance and to Dr. John J. Furth for his helpful criticism of the manuscript.

REFERENCES

Excretion of Spermidine from BHK-21/C13 Cells Exposed to 6-Thioguanosine

Maureen A. L. Melvin, William T. Melvin and Hamish M. Keir


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/38/9/3055

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.