Polyamines: Specific Metabolic Regulators or Multifunctional Polycations?


Received 28 July 1998

Regulation of polyamine acetylation and efflux in human cancer cells

H. M. Wallace and A. J. Mackarel

Departments of Medicine and Therapeutics and Biomedical Sciences, University of Aberdeen, Polwarth Building, Foresterhill, Aberdeen AB25 2ZD, Scotland, U.K.

Introduction

The polyamines spermidine, spermine and their diamine precursor putrescine, are normal and essential constituents of both prokaryotic and eukaryotic cells [1]. They are aliphatic polycations which are positively charged at physiological pH and are among the most cationic small molecules within the cell. Unlike the point charges of Mg²⁺ and Ca²⁺, the charge is distributed along the flexible carbon chain which enables the polyamines uniquely to bridge critical distances and thus allow specific interactions with polyanionic macromolecules such as DNA [2, 3]. Their high positive charge also prevents them from crossing biological membranes by simple diffusion.

The polyamines are required for optimal growth of almost all cell types and increased biosynthesis is necessary for the traverse of a cell through the cell cycle [4]. In contrast, depletion of polyamine content through inhibition of biosynthesis or via mutation of the key enzymes involved in their production results in significant inhibition of cell growth [5–7]. One of the most useful tools in this respect has been α-difluoromethylornithine, which is an enzyme-activated, irreversible inhibitor of ornithine decarboxylase, the rate-limiting enzyme in the biosynthetic pathway [8]. α-Difluoromethylornithine has been shown to be an effective inhibitor of tumour cell growth in culture [9] but trials in vivo have been less promising; cytopstatic rather than cytotoxic effects have been observed [10]. These disappointing results can be explained by the increased uptake of circulating polyamines that is induced to compensate for the decreased biosynthetic capacity. The circulating polyamines are provided in the diet, from intestinal flora and/or from tissue and cell destruction [7,11,12]. The ability of cells to reverse cellular polyamine depletion by recruitment of exogenous polyamines stimulated interest in characterizing the mechanism by which polyamine uptake occurs in cells.

Intracellular polyamine content in mammalian cells is regulated at three distinct levels: (i) biosynthesis through the two decarboxylase enzymes, ornithine and S-adenosylmethionine decarboxylases (for a review, see [13]); (ii) catabolism via the two step acetylation and oxidation pathway involving spermidine/spermine N′-acetyltransferase and polyamine oxidase (for a review see [14]); (iii) transport into and out of the cell (for reviews, see [15,16]). Of these processes, the one which has received the least attention is polyamine efflux or excretion. In the late 1970s it was shown that in response to a step down in growth rate, as a result of serum deprivation, BHK-21/C13 cells released significant amounts of radiolabelled polyamine into the culture medium [17]. Further analysis showed this to be a specific response in that the major excretory product was spermidine in either a free or conjugated form whereas the main intracellular polyamine was spermine [18]. This was shown to be a general response of these cells to a limitation in growth rate whether this was induced by contact inhibition of growth [19], nutrient deprivation [20] or treatment of the cells with cytotoxic drugs such as 5-fluorouracil, methotrexate...
[21,22] and 6-thioguanine [23]. The conjugated form of spermidine released was later identified as $N^1$-acetylspermidine, the product of $N^1$-acetyltransferase and the preferred substrate for polyamine oxidase [24].

It was important to establish at an early stage that the loss of polyamines from cells was not simply the result of leakage from damaged or dying cells. This was shown in three separate ways. Firstly, the cells actively releasing polyamines maintained a high viability as determined by the exclusion of the vital dye, Trypan Blue. Secondly, release of polyamines could be 'switched off' by inducing the cells to undergo a round of DNA synthesis and cell division; this indicated that the process was regulated. Thirdly the export was selective with the major polyamine outside the cell being different from the major polyamine within the cell [19,20]. In a kinetic study of polyamine turnover in mouse fibroblasts, McCormick [25] showed that dying cells do release polyamines but that it was almost exclusively spermine that was found in the extracellular medium of these cells. Interestingly the release of spermine showed a positive correlation with the release of $[^3]$H]DNA fragments, a classic sign of cell death.

Polyamines are also excretory products in man: significant amounts are found in urine [26]. Initial studies by the late Diane Russell [27] raised expectations that urinary polyamine measurements might prove to be of diagnostic use in cancer where elevated concentrations of acetylated and free polyamines were found. However a number of false positives in patients with psoriasis [28], and Duchenne muscular dystrophy [29] showed this to be inappropriate. Later evidence indicated that urinary polyamine measurements might still be useful but in monitoring the response of patients to therapy [30] and although this evidence still bears scrutiny there is little use made of these potentially valuable markers clinically. It is not clear from where the urinary polyamines in man, particularly in cancer patients, are derived but one suggestion is that they are released from tumour cells during the growth phase. An argument against this is the results from our group who examined the export of polyamines from normal and virus-transformed BHK cells and found that the transformed cells released less polyamine than the normal cells [31]. However, more recent studies have shown that human cancer cell lines do release polyamines in varying amounts [32,33].

The aim of this study was to investigate further the mechanism and regulation of polyamine export from human cancer cells using a cell culture model system.

Methods
The cell line used was HT115, which is derived from a human colonic carcinoma [34]. Cells were grown in DMEM supplemented with 10% horse serum, which is known to contain low levels of polyamine oxidase activity [35] under standard cell culture conditions (37°C, 5% CO$_2$:95% air). Intracellular polyamines were radiolabelled according to Wallace and Mackarel [36] using $[^3]$H]putrescine (0.5μCi/ml) for 36 h. The mono-layer was then washed twice in warmed DMEM and incubated in fresh unlabelled medium for 12 h. At this time the medium was changed and the treatments added ($t = 0$ h). Samples were collected at the appropriate times and polyamines determined in the cells and medium by liquid scintillation spectrometry and by HPLC [37].

Results
HT115 cells grew exponentially for approximately 144 h. They have a population doubling time of about 30 h and increases in cell number correlated with increases in protein content ($r^2 = 0.97$) and the latter was used as the major index of cell growth for this study. Putrescine, spermidine, spermine and $N^1$-acetylsperrmidine were routinely found in the cells in the order spermine > spermidine > putrescine > $N^1$-acetylsperrmidine.

In this type of radiolabelled tracer study it was essential to establish that the labelled polyamine distribution within the cells paralleled that of the free polyamines. In a direct comparison the proportion of each polyamine was approximately equal whether measured by HPLC or by liquid scintillation spectroscopy (Table 1). Analysis of polyamine export from the cells revealed an increase in extracellular polyamines with time; export at 96 h was approx. 4-fold greater than that at 0 h (Figure 1). Analysis of the distribution of radioactivity within the cells and in the extracellular medium showed that in the cells spermine predominated: on average its concentration was approx. twice that of spermidine (Figure 2). A small but significant amount of $N^1$-acetylsperrmidine (2%) was found in the cells. The pattern in the extracellular medium, however, was quite different: more than 80% of the
Table I

Comparison of polyamine distribution in HT115 cells: 
$[^{3}H]$ labelled polyamines versus total polyamine pool

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Total polyamines</th>
<th>Radiolabelled polyamines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>9.1 ± 1.8</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Spermidine</td>
<td>37.1 ± 1.5</td>
<td>35.2 ± 2.9</td>
</tr>
<tr>
<td>Spermine</td>
<td>51.0 ± 1.4</td>
<td>59.6 ± 1.4</td>
</tr>
<tr>
<td>N'-Acetylsperrmidine</td>
<td>2.8 ± 1.1</td>
<td>3.6 ± 0.5</td>
</tr>
</tbody>
</table>

Radiolabel was present as N'-acetylsperrmidine (Figure 2). The total polyamine content decreased over time, and was 25% less at 96 h than at 48 h.

Preliminary experiments to investigate the energy requirements of the export process using altered temperature showed a significant decrease in polyamine excretion after only 6 h incubation at the lower temperature. Efflux fell from $1.91 \pm 0.18 \times 10^5$ dpm/mg protein at 37°C to $0.54 \pm 0.04 \times 10^5$ dpm/mg protein at 4°C.

Discussion

It is clear from this study that human cancer cells do secrete polyamines constitutively at a significant rate (approximately 1.05 pmol/min per mg of protein) and that like BHK cells secretion is specific for N'-acetylsperrmidine (Figure 2). This is not an artefact of the experimental conditions because the distribution of radiolabelled polyamines reflects accurately that of the free polyamines measured by HPLC (Table 1). Neither is it the result of extracellular metabolism as incubation of individual polyamines with fresh medium or conditioned medium from cells after 96 h growth did not result in any metabolism of the polyamines (results not shown).
Acetylpolyamines may also be more lipophilic than the polyamines, which having negative log $D_{14}$ values will naturally partition into the aqueous phase and therefore be unlikely to pass through the lipid bilayer unassisted. The temperature dependence of the secretory process suggests that it requires energy although our attempts to prove this using inhibitors of oxidative phosphorylation have been unsuccessful. This indicates, therefore, that polyamine export is more likely to be a facilitated diffusion process that probably requires a protein carrier.

Polyamines are positively associated with high rates of cell growth and thus high concentrations are required in the early phase of culture. However, as cells in culture approach high cell densities their growth rate begins to slow and the need for polyamine growth factors decreases and therefore the polyamine content must decrease. This may be essential to prevent the cell committing suicide; it is well known that cells with an overproduction of growth or survival factors in the presence of a negative growth signal will enter the cell death programme known as apoptosis [40]. Indeed, our group has shown that excess polyamine can be cytotoxic to cells [41].

Our data are different from that of Tjandrawinata et al. [42,43] who showed that human leukaemic cells export significant amounts of putrescine but not of acetylpolyamines. The reason for these differences is not clear but it may reflect differences in polyamine metabolism within the cell types. For example, RAW 264 cells have high levels of membrane-bound amine oxidases which may metabolize the acetyl derivatives en route to the extracellular compartment. Alternatively, the cells may have differing amounts of N$^1$-acetyltransferase, the enzyme responsible for polyamine acetylation.

Human cancer cells contain higher polyamine concentrations than the equivalent normal cell [44] so is the amount excreted significant? HT115 cells contains approximately 4.5 mM polyamine 96 h after the end of the labelling period; losing 25% of this still leaves a significant amount of polyamine within the cell. Perhaps the role of this efflux is to maintain the total polyamine content below toxic levels [41]. The equivalent normal cells contain approx. one-quarter of this amount of polyamine [44] so the loss of 25% of the total polyamine could reduce the concentration to below 1 mM, which may be limiting in terms of cell growth and differentiation. If the amount of polyamine excreted by normal cells is compared with that excreted by malignant cells, it is shown that the former release more than 60% of their labelled polyamine over 96 h compared with only released by the latter 25%. This suggests that a decrease in the rate of efflux may be a contributory factor in the high concentrations of polyamines found in cancer cells compared with normal cells.

In summary, polyamine export appears to be a specific and regulated process which occurs constitutively in human colon cancer cells. It appears likely that this efflux contributes to the overall regulation of polyamine content in these cells probably in response to changes in the growth rate or density of the cells. In addition, the polyamines exported would be available for re-uptake and may be part of both autocrine and paracrine growth regulatory signals for these tumour cells.

We thank the University of Aberdeen Medical Endowments and Aberdeen Royal Hospitals N.H.S. Trust endowments for their financial support of this work.

Abbreviations used: ODC, ornithine decarboxylase; UTR, untranslated region.

The importance of the polyamines in cell function is reflected in a strong regulation of their intracellular levels [1–3]. Although it is likely that most of the specific functions of polyamines in the cell have not been identified yet, it is clear that too high concentrations of the larger polyamines are toxic to the cell, whereas too low concentrations have a negative effect on anabolic events such as the synthesis of DNA, RNA and protein in the cell [3]. Studies concerning polyamine metabolism have revealed a complex network of regulatory mechanisms involved in the cellular control of polyamine levels [2]. An adequate cellular polyamine homoeostasis is achieved by a careful balance between the synthesis, degradation and uptake of the amines. The degree to which the individual pathways contribute to polyamine homoeostasis may vary according to the situation.

Ornithine decarboxylase (ODC) catalyses the first and what is often considered to be the rate-limiting step in the biosynthesis of polyamines. Mammalian ODC is a highly regulated enzyme and the underlying mechanisms of this regulation are currently being studied [2,4–7]. Some of the regulatory mechanisms have been