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REPLACEMENT OF NATURAL POLYAMINES BY CADAVERINE AND ITS AMINOPROPYL DERIVATIVES IN EHRLICH ASCITES CARCINOMA CELLS

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SUMMARY: Ehrlich ascites carcinoma cells were cultured in the presence of difluoromethyl ornithine (DFMO) and micromolar concentrations of cadaverine for several months. This treatment resulted in a complete disappearance of putrescine and spermidine and reduced spermine content to traces of its normal content. The natural polyamines were replaced by cadaverine (about 40% of total polyamines), N-(3-aminopropyl)cadaverine (about 50%) and N,N'-bis(3-aminopropyl)cadaverine (about 5%). In comparison with untreated cells or cells grown in the presence of DFMO and putrescine, the "cadaverine cells" grew definitely slower, their protein synthesis was depressed while DNA and RNA syntheses proceeded at near normal rate. In spite of the high intracellular concentrations of cadaverine and its aminopropyl derivatives, the tumor cells grown in the presence of DFMO and cadaverine, behaved exactly like cells severly depleted of putrescine and spermidine. Though exposed to DFMO, ornithine decarboxylase activity was almost 10 times higher than that in untreated cells. S-Adenosyl-L-methionine decarboxylase activity was likewise strikingly elevated, and these cells transported methylglyoxal bis(guanylhydrazone) (MGBG) at a rate that was more than 5 times faster than that in untreated cells. Furthermore, these cells exhibited arginase activity, which was less than one fifth of that found in untreated cells.

The natural polyamines putrescine, spermidine and spermine are apparently required for normal cell proliferation as an inhibition of their accumulation in animal cells leads to a more or less complete cease of cell division (for ref. see 1). In some cases the requirements for the natural polyamines can apparently be largely fulfilled by closely related amines such as 1,3-diaminopropane or cadaverine at least for short periods of time (2,3).

The capability of certain diamines to replace the natural polyamines in supporting growth may conceivably be related to their ability to serve as substrates for spermidine and spermine synthases thus giving rise to the formation of analogs of the higher polyamines. Cadaverine, acts as a substrate for spermidine synthase though at a rate of only 3 to 10% of that of putrescine (4,5), and is converted to N-(3-aminopropyl)cadaverine and N,N'-bis(3-aminopropyl)cadaverine in Ehrlich ascites carcinoma cells exposed to DFMO (6).

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By exposing cultured Ehrlich ascites carcinoma cells to DFMO, an irreversible inhibitor of ornithine decarboxylase (7) in the presence of cadaverine, we have succeeded to replace the natural polyamines by cadaverine-derived amines to an extent that no putrescine or spermidine and only traces of spermine remained. The new polyamine pattern appeared to be compatible with life, since these cells continued to grow, albeit slowly, for several months, yet they continued to express changes that are typical to polyamine depleted cells.

MATERIALS AND METHODS

Cell cultures: Ehrlich ascites carcinoma cells were grown in suspension cultures in RPMI 1640 medium supplemented with 5% pooled human serum and antibiotics (3). For replacement of the natural polyamines, the cultures were grown in the presence of 5 mM DFMO and 0.005 mM cadaverine (or 0.005 mM putrescine in the control cultures).

Materials: Difluoromethyl ornithine was a generous gift from the Centre de Recherche Merrell International (Strasbourg, France). MGBG was obtained from Orion Pharmaceutical Company (Helsinki, Finland). [^{3}H]Thymidine (sp. act. 24 Ci/mmol), [^{14}C]leucine (sp. act. 330 Ci/mol), [^{3}H] uridine (sp. act. 46 Ci/mmol) and [U-^{14}C]arginine (sp. act. 344 Ci/mol) were purchased from the Radiochemical Centre (Amersham, Bucks., England).

Analytical methods: Polyamines were determined by the method of Seiler (8) as modified by Dreyfuss et al. (9). Protein was determined by the method of Lowry et al. (10), DNA by the method of Giles and Myers (11) and RNA according to Ashwell (12).

The activities of ornithine decarboxylase (13) and adenosylmethionine decarboxylase (14) were measured by published methods. MGBG was determined by the method of Seppänen et al.(15).

For the determination of arginase activity the following method was developed. Cytosol fraction (0.125 ml) obtained from the carcinoma cells was incubated in 50 mM glycine buffer (pH 9.75) containing 1 mM $MnCl_2$, 2.5 mM L-arginine and 0.5 μCi of L-[U-^{14}C]arginine. The final volume of the incubation mixture was 0.15 ml. After an incubation of 1 h at 37°C, the reaction was halted with 0.015 ml of 50% trichloroacetic acid containing 0.5 mM L-ornithine as carrier. Thirty microliters of the supernatant fraction was subjected to paper electrophoresis using 0.065 M sulphosalisylic acid (pH 3.2) as the buffer. The electrophoresis was continued for 3 h at 300 V. After staining with ninhydrin, the ornithine fraction was cut off and counted for radioactivity.

RESULTS

When Ehrlich ascites carcinoma cells were cultured in the presence of DFMO and cadaverine (but not in its absence) they continued to grow for several months. Table 1 shows the polyamine pattern of such cells in comparison with untreated tumor cells and cells continuously exposed to DFMO in the presence of putrescine. While the polyamine pattern of the latter cells was fully comparable with that of untreated cells, the cadaverine-treated tumor cells did not contain putrescine or spermidine and only traces of spermine were present (Table 1). The natural polyamines were almost

Table 1. Polyamine concentrations in untreated Ehrlich ascites cells and in cells exposed to DFMO and putrescine or cadaverine. Polyamine concentrations were measured at 24 h after dilution of the cultures. Other details as in Fig. 1. Means ± standard deviations are given. Put, putrescine; Cad, cadaverine; Spd, spermidine; Apc, aminopropylcadaverine; Spm, spermine; Bis(ap)c, N,N'-bis(aminopropyl)cadaverine; n.d., not detected.

Treatment	Put	Cad	Spd (nmol/mg DNA)	Apc	Spm	Bis(ap)c
None	92 ± 20	n.d.	344 ± 99	n.d.	172 ± 72	n.d.
DFMO + Put	80 ± 6	n.d.	299 ± 19	n.d.	185 ± 20	n.d.
DFMO + Cad	n.d.	286 ± 35	n.d.	385 ± 27	57 ± 6	46 ± 1

totally replaced by cadaverine and its aminopropyl derivatives, mainly N-(3-aminopropyl)cadaverine.

The growth of cadaverine cells was clearly slower than that of cells containing normal polyamine pattern (Fig.1). This slow growth was also reflected in the synthesis of macromolecules. While the synthesis of RNA and DNA was decreased by only about 20%, the incorporation of labeled leucine into protein was depressed by 50% (Table 2).

The most striking differences between the three types of cultures were found in the activities of ornithine and adenosylmethionine decarboxylases

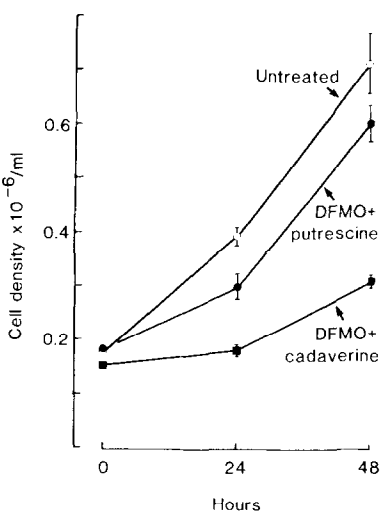

Fig. 1. Growth of untreated Ehrlich ascites carcinoma cells and of cells exposed to DFMO in the presence of putrescine or cadaverine. The tumor cells, except untreated cells, were continuously grown in the presence of 5 mM DFMO and 5 μM putrescine or cadaverine for more than 4 months. The cell densities were measured at 24 and 48 h after medium change and dilution. Means from 3 to 4 cultures ± standard deviations are given.

Table 2. Rates of protein synthesis, DNA synthesis and RNA synthesis in untreated Ehrlich ascites cells and in cells exposed to DFMO and putrescine or cadaverine. Experimental details as in Table 1.

Treatment	Protein synthesis cpm/μg protein	DNA synthesis cpm/mg DNA	RNA synthesis cpm/μg RNA
None	32.9 ± 1.3	2.67 ± 0.23	90.6 ± 3.0
DFMO + Put	29.2 ± 0.7 **	2.22 ± 0.16 *	88.6 ± 3.5
DFMO + Cad	17.6 ± 0.8 ***	2.10 ± 0.18 *	69.5 ± 9.6 **

***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$

(Table 3). Tumor cells grown in the presence of DFMO and cadaverine exhibited ornithine decarboxylase activity (measured after sufficient dilution or dialysis) that was almost 10 times higher than that in untreated cells. This finding indicates that in the absence of the natural polyamines, the tumor cells overproduced ornithine decarboxylase, a phenomenon earlier observed when hepatoma cells were exposed to methylornithine, a reversible inhibitor of ornithine decarboxylase (16).

Similarly, the activity of adenosylmethionine decarboxylase was greatly elevated in cells exposed to DFMO and cadaverine (Table 3), a phenomenon typical to polyamine depleted cells (2,17,18).

Another characteristic of polyamine-starved cells is their enormous capacity to transport exogenous polyamines and MGBG into the cell's interior (19). In spite of the high intracellular concentrations of cadaverine and aminopropylcadaverine, the cells exposed to DFMO in the presence of cadaverine fully retained the enhanced uptake of MGBG that was about 6 times more rapid than that in untreated cells or tumor cells exposed to DFMO in the presence of putrescine: i.e. the former cells

Table 3. Activities of ornithine decarboxylase and adenosylmethionine decarboxylase in Ehrlich ascites cells. Experimental details as in Table 1.

Treatment	Ornithine decarboxylase (pmol/mg protein/h)	Adenosylmethionine decarboxylase
None	262 ± 54	1245 ± 59
DFMO + Put	129 ± 11 **	1392 ± 63
DFMO + Cad	2380 ± 660 ***	11360 ± 960 ***

***, $p < 0.001$; **, $p < 0.01$

Table 4. Arginase activity in untreated Ehrlich ascites cells and in cells continuously exposed to 5 mM DFMO in the presence of 5 μM putrescine or cadaverine. Enzyme activities were determined at 48 h after dilution of the cultures.

Treatment	Arginase activity (nmol/mg protein/h)
None	11.26
DFMO + Putrescine	11.75
DFMO + Cadaverine	1.64

attained an intracellular MGBG concentration of 4.09 mM in 4 h when exposed to MGBG in comparison with 0.63 and 0.60 mM found in cells with normal polyamine pattern.

As already shown in Table 3, cadaverine containing cells exibited strikingly elevated ornithine decarboxylase activity, yet no putrescine or spermidine and only traces of spermine were found in these cells (Table 1). This apparent discrepancy gets its explanation in Table 4 showing that a long-term exposure to DFMO and cadaverine led to an almost complete disappearance of arginase activity.

DISCUSSION

The view that undisturbed accumulation of the natural polyamines is required for proper animal cell proliferation has been greatly strengthened during last few years when potent and specific inhibitors of polyamine biosynthesis have become available (1). There exists, however, only fragmentary information as regards the structural specificity of various amine compounds to fulfil the requirements for putrescine, spermidine and spermine.

Our results indicate that a replacement of putrescine and spermidine by their closely related analogs cadaverine and N-(3-aminopropyl)cadaverine is compatible with life, at least in Ehrlich ascites carcinoma cells.

Even though cadaverine and aminopropylcadaverine appear to meet the minimal requirements for cell proliferation, these amines did not act as intracellular regulatory molecules as regards the repression of compensatory changes induced by putrescine and spermidine depletion. Thus in the presence of even high concentrations of cadaverine-derived amines, the tumor cells continued to overproduce ornithine decarboxylase. McCann et al. (16) reported that rat hepatoma cells exposed to methylornithine exhibited greatly elevated ornithine decarboxylase activity. Although stabilization of the enzyme by the inhibitor against intracellular proteolysis was probably the major reason for the increased enzyme

activity, putrescine and spermidine depletion most likely also enhanced the synthesis of the enzyme protein.

Quite unexpected, however, was our finding that greatly increased levels of ornithine decarboxylase activity could be found in tumor cells exposed to DFMO, an irreversible enzyme-activated inhibitor of the enzyme (7). Although there is no doubt that the enzyme is irreversibly inactivated in vitro when exposed to DFMO, the situation may be different in vivo, especially under conditions where ornithine decarboxylase is expressed at an accelerated rate. Under these circumstances DFMO may in the first hand act as a competitive inhibitor of the enzyme.

The induction of adenosylmethionine decarboxylase by polyamine depletion is a well documented phenomenon (2,17,18,20), as is the striking enhancement of polyamine/MGBG transport in response to DFMO (19,20). As shown here, both these compensatory mechanisms remained operating in spite of high intracellular concentrations of cadaverine and N-(3-aminopropyl)-cadaverine.

Another interesting characteristic of the cadaverine and aminopropyl-cadaverine containing cells was the profound reduction of arginase activity. There are several pieces of evidence indicating that arginase acts, in tissues which are not engaged with urea production, in fact as a polyamine biosynthetic enzyme (21,22) and may be coordinately induced together with the two decarboxylases (21,22). The present results, representing an inverse situation, i.e. a disappearance of the natural polyamines, may be taken as a further evidence supporting a regulatory involvement of arginase in the biosynthesis of polyamines.

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