DISSOCIATION OF THE EARLY ANTIPROLIFERATIVE ACTION OF METHYLGLYOXAL BIS(GUANYLHYDRAZONE) FROM POLYAMINE DEPLETION

A comparison of the effects of DL-α-difluoromethyl ornithine and methylglyoxal bis(guanylhydrazone) on the growth of human fibroblasts

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1. Introduction

Association of polyamines with cell growth appears to be a general phenomenon [1–3]. The mechanism of action of these compounds is, however, still largely obscure. This problem has been recently approached by attempts to block polyamine synthesis with various inhibitors [3]. Methylglyoxal bis(guanylhydrazone) (MGBG) which prevents the synthesis of spermidine and spermine by blocking the decarboxylation of adenosylmethionine [4] retarded DNA synthesis in concanavalin A-activated bovine lymphocytes at low concentrations sufficient to prevent polyamine synthesis but did not affect RNA or protein synthesis [5]. Inhibition of DNA synthesis following exposure to MGBG has also been observed in cultures of human fibroblasts WI-38 [6] and HeLa cells [7]. In addition, rat embryo fibroblasts [8] and rat brain tumor cells [9] were reportedly arrested in G_1 after the inhibitor treatment. The results obtained with this inhibitor, however, should be interpreted with certain reservations, since in leukemia L 1210 cells the drug has been reported to cause early cytotoxic effects which may be unrelated to its effects on polyamine metabolism [10].

A newly discovered irreversible inhibitor of ornithine decarboxylase, DL-α-difluoromethyl ornithine (DFMO), prevented the accumulation of putrescine and spermidine in rat hepatoma cells, mouse leukemia cells and human prostate adenoma cells, but the growth was not retarded until after a delay of one cell generation [11].

The present study compares the temporal sequence of polyamine depletion and inhibition of macromolecular synthesis following addition of MGBG or DFMO to cultured human embryonic fibroblasts. The results show that the prevention of polyamine accumulation of DFMO during the first day did not immediately affect the rate of DNA synthesis. In contrast, MGBG, at μM levels, caused a marked inhibition of the synthesis of DNA and protein as early as 12 h after addition of the drug. At that time the accumulation of polyamines proceeded as in the absence of the inhibitor. The initial inhibition of the growth of human fibroblasts by MGBG thus may not be primarily mediated by polyamine metabolism.

2. Materials and methods

2.1. *Cells*

Human embryo skin fibroblasts were cultured in 30 mm or 90 mm plastic Petri dishes in MEM [12] supplemented with 10% pooled human serum unless otherwise stated. Before use the cultures were incubated overnight without serum.

2.2. *Chemicals*

L-[U-^{14}C]leucine (spec. radioact. 354 mCi/mmol), [6-^{3}H]thymidine (26.4 Ci/mmol) and [5-^{3}H]uridine

(27 Ci/mmol) were purchased from the Radiochemical Centre (Amersham). DL-α-difluoromethyl ornithine was a generous gift from Centre de Recherche Merrell International (Strasbourg)

2.3. *Analytical methods*

For analysis of polyamine content the fibroblasts were collected by centrifugation after trypsinization (0.25% trypsin) of the dishes. Polyamines were assayed by the dansylation method as in [13].

The rates of DNA, RNA and protein synthesis were determined by labeling the cultures for 60 min with radioactive thymidine (1 μCi/ml), uridine (2 μCi/ml) and leucine (0.1 μCi/ml). Determination of the macromolecules was made as in [13]. For analysis of the percentage of the labeled cells, cultures were incubated with [^{3}H]thymidine (2 h) and counted after autoradiography as in [12]. The number of cells was determined with an electronic counter [12].

For the assay of thymidine kinase the fibroblasts were washed with 0.9% NaCl and suspended in 0.75 ml 25 mM Tris–HCl buffer (pH 7.4) containing 1 mM dithiothreitol. The cells were disrupted by sonication and the thymidine kinase (EC 2.7.1.21) activity was measured by the method in [14].

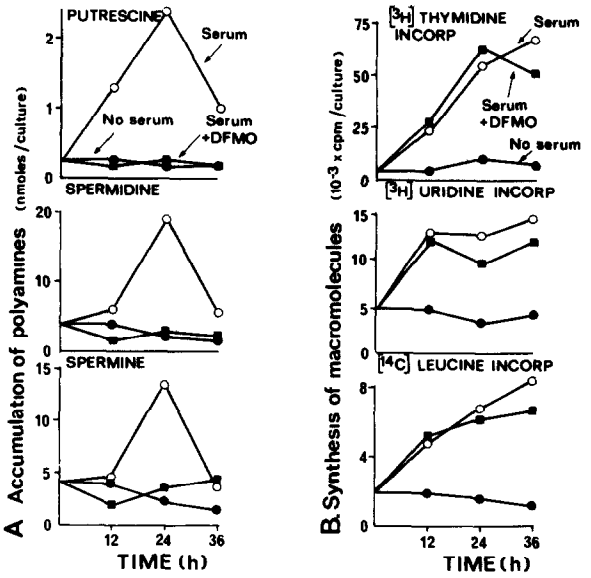

Fig 1. Effect of difluoromethyl ornithine (DFMO) on the accumulation of polyamines (A) and on the synthesis of DNA, RNA and protein (B) in human fibroblasts after serum stimulation. Sparsely populated cultures (7 μg DNA/culture) were synchronized by serum starvation and grown in the absence (●——●) or presence of 10% human serum without (○——○) or with 1 mM DFMO (■——■) for the indicated times. For the other culture conditions and measurement of polyamines and synthesis of macromolecules see section 2.

3. Results

Stimulation of the serum-starved human fibroblasts with a fresh medium supplemented with 10% homologous serum resulted in several-fold increases in the levels of putrescine, spermidine and spermine (fig.1A) and in a simultaneous activation of the synthesis of DNA, RNA and protein (fig.1B). Addition of DFMO (1 mM) to the cultures almost totally abolished the accumulation of all the polyamines during the first 24 h of stimulation, but the synthesis of macromolecules was not greatly disturbed up to 36 h (fig.1).

In contrast, MGBG (5 μM) caused a pronounced inhibition of the incorporation of both [^{3}H]thymidine into DNA and [^{14}C]leucine into protein as early as 12 h after the simultaneous addition of serum and the compound (fig.2). Inhibition of RNA synthesis by the drug was less conspicious. It is noteworthy that the accumulation of polyamines at 12 h was reduced to lesser extent by MGBG than by DFMO, and yet the synthesis of DNA was markedly prevented only in the presence of the former inhibitor (fig.3). Exogenous spermidine and spermine but not putrescine (10 μM) largely normalized the synthesis of DNA after MGBG treatment (results not shown). It is possible, however, that the higher polyamines brought about this reversal by interfering with the MGBG transport [15]. The enhancement of the activity of thymidine kinase at 24 h after serum addition was totally abolished by MGBG, whereas DFMO only slightly decreased the enzyme activity (table 1).

The growth-inhibitory effect of DFMO on human fibroblasts appeared to depend on the extent of the stimulation of cellular growth. Slowly-growing cells, e.g. in the presence of low concentrations of serum, were less sensitive to the drug (table 2) A difference in the total number of cells in the DFMO-treated and control cultures was not observed until after

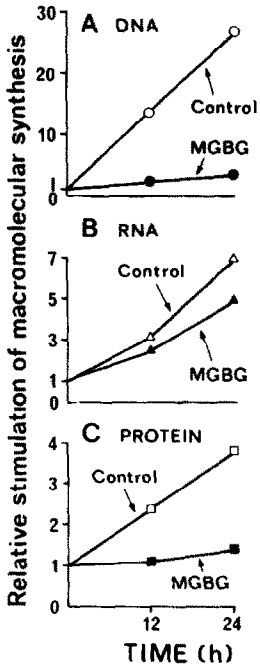

Fig.2 Effect of methylglyoxal bis(guanylhydrazone) (MGBG) on the synthesis of DNA, RNA and protein in serum-stimulated fibroblasts. MGBG (5 μM) was added simultaneously with 10% human serum to the serum-starved cultures. The synthesis of the macromolecules are expressed as relative stimulations brought about by serum addition

Table 1
Effect of difluoromethyl ornithine and methylglyoxal bis(guanylhydrazone) on the activity of thymidine kinase in serum-stimulated human fibroblasts

Additions	Activity of thymidine kinase (nmol/30 min)	
	per mg DNA	per mg soluble protein
No serum	9 16	0 81
10% serum	54 8	2 95
10% serum + DFMO	44.7	2.41
10% serum + MGBG	11 9	0 65

Fibroblast cultures (14 μg DNA/culture at the start of the experiment) were stimulated with 10% human serum in the absence or presence of difluoromethyl ornithine (DFMO, 1 mM) or methylglyoxal bis(guanylhydrazone) (MGBG, 5 μM) for 24 h Thereafter the activity of thymidine kinase was determined as described in section 2

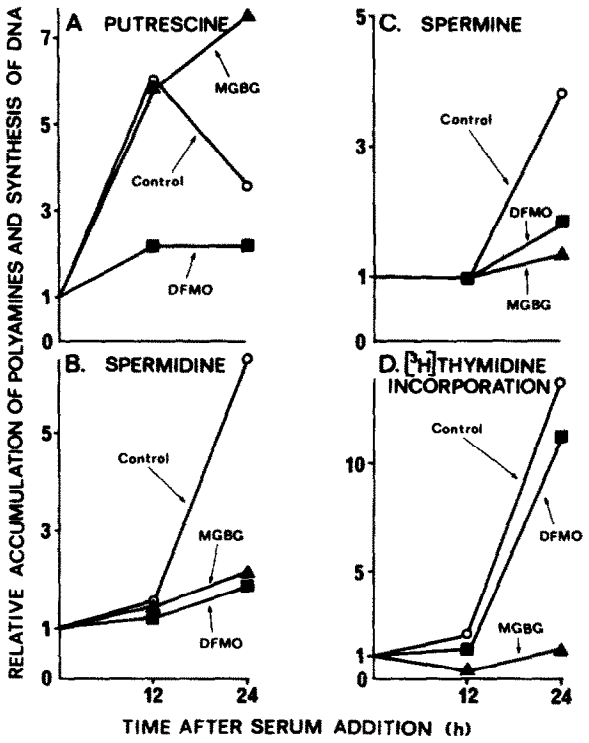

Fig.3. Effect of difluoromethyl ornithine (DFMO) and methylglyoxal bis(guanylhydrazone) (MGBG) on the accumulation of putrescine (A), spermidine (B) and spermine (C) as well as on the incorporation of [^{3}H]thymidine into DNA (D) during the first 24 h after stimulation of the serum-starved fibroblasts with 10% human serum. DFMO (1 mM) and MGBG (5 μM) were added simultaneously with the serum to the sparsely populated cultures (7 μg DNA/culture) The results are expressed as relative values in comparison to cultures lacking serum

2 days (fig.4). The DFMO-induced retardation of cell proliferation was reversed by exogenous putrescine (fig.4). The inhibition of the incorporation of [^{3}H]thymidine into DNA by DFMO likewise became more pronounced with increasing time of exposure; after 72 h the synthesis of DNA was decreased by 90% (table 3). Putrescine completely reversed this inhibition (table 3). Higher polyamines could also restore the macromolecular synthesis after the treatment with DFMO (results not shown).

Table 2
Inhibition of DNA synthesis by difluoromethyl ornithine in human fibroblasts after stimulation with different concentrations of serum

Incubation time (h)	Additions	[^{3}H]Thymidine incorp (cpm)	Inhibition (%)	Proportion of labeled cells (%)	Inhibition (%)
48	2% serum	3780		3.9	
48	2% serum + DFMO	3720	(2)	3.4	(13)
48	10% serum	6120		7.8	
48	10% serum + DFMO	3600	(41)	5.5	(30)
72	2% serum	4800		1.9	
72	2% serum + DFMO	3780	(21)	0 8	(58)
72	10% serum	9700		7 3	
72	10% serum + DFMO	3740	(61)	0.7	(90)

After serum starvation of the fibroblasts the old medium was replaced with a fresh one containing either 2% or 10% serum. The concentration of difluoromethyl ornithine (DFMO) was 1 mM Percentage of the labeled cells was determined as described in section 2

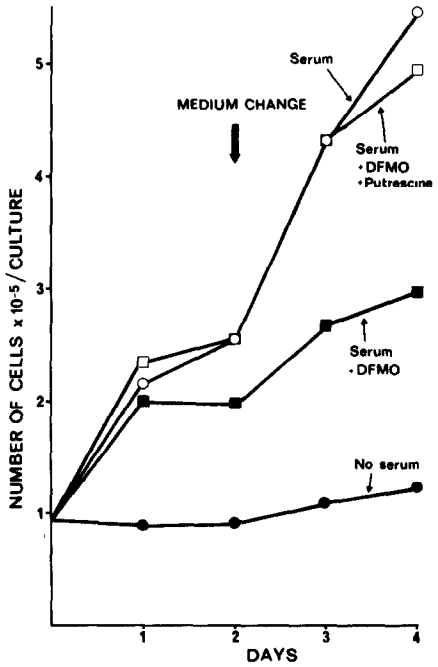

Fig.4. Effect of difluoromethyl ornithine (DFMO) on the increase in cell number in response to serum addition Serum-starved human fibroblasts were grown in the absence or presence of 10% human serum without or with 1 mM DFMO alone or combined with 10 μM putrescine. After 2 days of incubation the old medium was replaced with a fresh one containing the same ingredients as the old medium The number of cells at the indicated times was determined using an electronic cell counter

4. Discussion

The importance of polyamines for cell growth is indicated by the experimental findings showing that the growth rate of polyamine-deficient bacterial [16] and yeast [17,18] mutants as well as animal cells depleted of polyamines with specific inhibitors [3], is clearly retarded. Furthermore, the growth of some cell lines is stimulated upon addition of exogenous polyamines [19,20].

This report shows that although both DFMO and MGBG effectively prevented polyamine accumulation in cultured human embryonic fibroblasts stimulated to

Table 3
Reversal by putrescine of the difluoromethyl ornithine-induced inhibition of DNA synthesis

Additions	[^{3}H]Thymidine incorporation	
	at 24 h (cpm)	at 72 h (cpm)
No serum	560	120
10% serum	18 100	7100
10% serum + DFMO	14 300	910
10% serum + DFMO + putrescine	17 000	7800

See the legend to fig 4 for experimental details

proliferate, the time-course of their inhibition of the synthesis of DNA, RNA and protein was strikingly different. The early inhibitory effect of MGBG on the macromolecular synthesis at 12 h in the absence of a respective depletion of polyamines strongly suggests that inhibitory mechanisms unrelated to polyamine metabolism are involved. The significance of polyamine depletion in the growth retardation of mouse L 1210 cells after MGBG treatment has similarly been questioned [21]. Whether this inhibition is related to an early mitochondrial damage recently reported in L 1210 cells [10] is not known. It is conceivable that the early antiproliferative effect of MGBG on human fibroblasts could result from a primary fall in protein synthesis (fig.2C), first manifested by the decrease in the activities of enzymes turning over rapidly like thymidine kinase [22] (table 1), and be secondarily reflected by impaired DNA synthesis due to a deficient uptake/phosphorylation of the labeled thymidine. This kind of mechanism of action has been recently proposed for MGBG in mitogen-activated guinea pig lymphocytes [23].

Prevention of the accumulation of putrescine and spermidine but not that of spermine by DFMO in several malignant cell lines has been reported to result in an inhibition of cell proliferation after a delay of one generation period [11]. In agreement with these results, we found that the inhibition of macromolecular synthesis and proliferation of human fibroblasts by DFMO did not take place during the first 24 h in spite of almost total block of the accumulation of all the polyamines. The delayed inhibition of macromolecular synthesis after DFMO treatment gives us reason to speculate that polyamines may participate in the biogenesis of some cellular component(s) which are required in the replication, and the surplus of which is found in quiescent fibroblasts. Depletion of polyamines would thus influence the process of replication only after the supply of the postulated component(s) becomes limiting. This idea supported by the relative ineffectiveness of DFMO in slowly growing cultures. Human fibroblasts whose growth is temporarily arrested by serum starvation differ from nonproliferating human lymphocytes in their response to DFMO upon stimulation. In contrast to fibroblasts, DFMO inhibited the macromolecular synthesis of phytohaemagglutinin-stimulated lymphocytes already in the first cell cycle [13]. This might be due to a lack or shortage of the above-hypothesized cellular components in the lymphocytes. In any case, the proper progression of cell proliferation appears to be polyamine-dependent, by a mechanism which still awaits to be elucidated.

Acknowledgements

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