Cadaverine supplementation during a chronic exposure to difluoromethylornithine allows an overexpression, but prevents gene amplification, of ornithine decarboxylase in L1210 mouse leukaemia cells

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We recently selected a variant mouse L1210 leukaemia-cell line overproducing ornithine decarboxylase (ODC) (EC 4.1.1.17) as a result of chronic exposure to 2-difluoromethylornithine (DFMO) in the presence of micromolar concentrations of cadaverine. These cells, now grown for more than 2 years in the presence of DFMO and cadaverine, continued to accumulate ODC-specific mRNA in an amount 30–50 times higher than that in the parental cells, yet showing practically no changes in the gene dosage for the enzyme. However, analysis of the genomic DNA with the isoschizomeric restriction enzymes *HpaII* and *MspI* revealed that the ODC sequences in the overproducer cells were hypomethylated in comparison with the parental cells. The natural polyamines (putrescine, spermidine and spermine) were almost totally replaced by cadaverine and aminopropylcadaverine. Omission of cadaverine from the culture medium, but leaving 10 mM-DFMO, resulted in an about 10-fold ODC gene amplification within a few weeks. The accumulation of ODC mRNA was enhanced by the same factor. Concomitantly, the content of the natural polyamines was almost normalized, representing about 65% of that found in the parental cells. The present results suggest that, under a given selection pressure, an overproduction of the target gene product may be primarily based on an enhanced transcriptional activity, possibly associated with hypomethylation and, if not sufficient, a secondary amplification of the active gene occurs.

INTRODUCTION

The development of resistance to DFMO upon chronic exposure seems to involve several different mechanisms. In addition to the 'conventional' gene amplification in mouse (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985a) and in human (Leinonen *et al.*, 1987) tumour cells, an overproduction of ODC can occur in the absence of any increase in the gene copy number. We found (Alhonen-Hongisto *et al.*, 1985b) that an exposure of mouse L1210 leukaemia cells to DFMO in the presence of micromolar concentrations of cadaverine led to a strikingly enhanced expression of ODC, with minimum changes in the gene dosage of the enzyme. Similarly, McConlogue *et al.* (1986) showed that an overproduction of ODC can be based on an enhanced accumulation of mRNA in the absence of any gene amplification, or even on a more efficient translation of normal amounts of the message. Human tumour cells can also acquire resistance to DFMO without overproducing ODC, as we recently selected a DFMO-resistant human myeloma cell line that did not overproduce ODC but displayed a strikingly elevated arginase (EC 3.5.3.1) activity, which was probably responsible for a continuous production of the natural polyamines in the presence of millimolar concentrations of DFMO (Alhonen-Hongisto *et al.*, 1987). In all of the cited cases, the basis for the resistance to DFMO has been a substantial production of the natural polyamines. However, more puzzling was the observation by Medrano *et al.*

(1986), indicating that a brief exposure to cycloheximide rendered several human and mouse cell lines resistant to DFMO in the absence of any significant polyamine production. The latter authors proposed that the resistance was acquired by an expression of other growth-related genes fulfilling the requirements for the polyamines.

We have now grown for more than 2 years a L1210 mouse leukaemia-cell line in the presence of 10 mM-DFMO and 5 μ M-cadaverine, which overexpresses ODC without any appreciable gene amplification. Omission of cadaverine, but retaining DFMO, rapidly led to an amplification of ODC genes associated with an accumulation of the enzyme's mRNA up to 500-fold higher than that found in the potential cells.

EXPERIMENTAL

Cell cultures and selection of the variants

Mouse L1210 leukaemia cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) pooled human serum (Finnish Red Cross Transfusion Service, Helsinki, Finland) and antibiotics (penicillin and streptomycin). The DFMO-resistant cells were cultured in the presence of 10 mM-DFMO and 5 μ M-cadaverine for more than 2 years. This cell line was designated as L1210/10DC. After about 2 years a further DFMO-resistant cell line was selected by the removal of

cadaverine from the L1210/10DC cells. This cell line was designated as L1210/10D.

Materials

DFMO was generously given by Centre de Recherche Merrell International (Strasbourg, France). DL-[1-¹⁴C]-Ornithine (sp. radioactivity 61 Ci/mol) and 5'-[α -³²P]-dCTP (sp. radioactivity 410 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). For the hybridization studies, a cDNA clone (pODC16) (Kontula *et al.*, 1984; Jänne *et al.*, 1984), kindly provided by Dr. O. A. Jänne, was used.

Analytical and preparative methods

Polyamines were determined by the method of Hölttä *et al.* (1979) and the activity of ODC was measured by the method of Jänne & Williams-Ashman (1971). Genomic DNA was isolated by the method of Blin & Stafford (1976). DNA was digested with *Eco*RI, *Hpa*II or *Msp*I (Amersham International) restriction endonucleases, electrophoresed in 0.9%-agarose gels, transferred to nitrocellulose filters (Southern, 1975) and hybridized to nick-translated (Rigby *et al.*, 1977) pODC16 (sp. radioactivity 1×10^8 c.p.m./ μ g). Total cellular RNA was isolated by the LiCl/urea method of Auffray & Rougeon (1980), size-fractionated by electrophoresis in 1.4%-agarose gels in the presence of 1 M-glyoxal, transferred (Thomas, 1980) to Gene-Screen membranes (New England Nuclear Corp.) and hybridized to nick-translated pODC16. For the cyto-dot analyses of the ODC mRNA amounts, the tumour cells were lysed in 10 mM-Tris/HCl (pH 7.5)/1 mM-EDTA buffer containing 1% Nonidet P-40. The lysates were applied with a manifold (Bethesda Research Laboratories, Gaithersburg, MD, U.S.A.) to a polyuridylylated-mRNA affinity paper (Hybond-mAP; Amersham International) under loading and washing conditions described by Werner *et al.* (1984).

RESULTS

We have been growing a L1210 mouse leukaemia-cell line in the presence of 10 mM-DFMO and 5 μ M-cadaverine for more than 2 years. These variant cells continued to overproduce ODC by virtue of strikingly enhanced accumulation of mRNA for the enzyme, with minimal changes of the gene dosage (Alhonen-Hongisto *et al.*, 1985b).

As depicted in Fig. 1, the cells adapted to grow in the presence of 10 mM-DFMO and 5 μ M-cadaverine (L1210/10DC) grew as well as the parental (L1210) cells. Similarly, an addition of DFMO (10 mM) with cadaverine to the parental cells (L1210 + DFMO + cadaverine) did not inhibit cell growth, whereas 10 mM-DFMO alone totally blocked the proliferation. The leukaemia cells from which cadaverine was removed (still retaining 10 mM-DFMO) about 6 weeks before the experiment (L1210/10D) grew, albeit slower than the parental cells.

Determination of ODC activities (Table 1) revealed that the leukaemia cells chronically exposed to DFMO in the presence of cadaverine (L1210/10DC) exhibited about 20 times higher enzyme activity than the parental cells (L1210). The removal of cadaverine, but retaining DFMO (L1210/10D), led to a further increase in ODC

Fig. 1. Growth of parental L1210 cells without or with the addition of 10 mM-DFMO alone or with 5 μ M-cadaverine, and of the variant cells chronically exposed to 10 mM-DFMO and 5 μ M-cadaverine (L1210/10DC), and of cells derived from the latter cell line from which cadaverine was removed 6 weeks before the experiment (L1210/10D).

Results are means $\pm$ S.D. (vertical lines) for triplicate cultures.

Table 1. ODC activity in untreated L1210 cells (L1210), in cells continuously exposed to DFMO and cadaverine (L1210/10DC) and in cells from which cadaverine was removed 6 weeks before the experiment (L1210/10D)

Results are means $\pm$ S.D. for triplicate cultures.

Cell line	ODC activity (pmol/60 min per 10^6 cells)
L1210	18.1 ± 4.2
L1210/10DC	368 ± 32
L1210/10D	1900 ± 330

activity, now being more than 100-fold in comparison with the parental cells (Table 1).

As shown in Fig. 2 (*Eco*RI digestion) (lanes 1 and 2), the gene dosage for ODC has remained the same in the variant cell line selected in the presence of DFMO and cadaverine (see also Alhonen-Hongisto *et al.*, 1985b). Thus the presence of cadaverine somehow allowed an overexpression of ODC in DFMO-treated cultures, but prevented the gene amplification. Lane 3 in Fig. 2 represents the situation at 6 weeks after the removal of

Fig. 2. Restriction-enzyme (*EcoRI*) analysis of ODC sequence in genomic DNA

DNA was isolated from untreated L1210 cells (lane 1), from cells continuously exposed to DFMO and cadaverine (lane 2) and from cells from which cadaverine was removed 6 weeks before the analysis (lane 3). DNA (10 μ g) was digested with *EcoRI* restriction endonuclease, size-fractionated by electrophoresis and hybridized with nick-translated pODC16.

cadaverine from DFMO-treated L1210 leukaemia cells. As shown, the genomic ODC sequences were distinctly amplified (probably more than 10-fold). A restriction-enzyme analysis with the isoschizomers *HpaII* and *MspI* (both cleaving at 5'-CCGG-3'; *HpaII* only when the internal cytosine is not methylated) is depicted in Fig. 3. As shown, the ODC sequences and/or sequences flanking them in L1210/10DC cells were distinctly less methylated (smaller *HpaII* restriction fragments) than those in the parental cells (L1210). Interestingly, the amplified sequences in L1210/10D cells possessed exactly the same methylation pattern seen in L1210/10DC cells indicating that the hypomethylated status was retained during the gene amplification in response to the removal of cadaverine.

Fig. 4 depicts a Northern-blot analysis from the same cells, i.e. parental cells (lane 1), cells treated continuously with DFMO and cadaverine (lane 2) and DFMO-cadaverine-treated cells at 6 weeks after the removal of cadaverine (lane 3). Thus the gene amplification upon removal of cadaverine, shown in Fig. 2, was associated with strikingly enhanced accumulation of the ODC mRNA.

We next attempted to quantify the changes occurring in the accumulation of ODC mRNA. Fig. 5 shows a cyto-dot analysis of the amounts of ODC-specific mRNA at 4 and 24 h after dilution of the cultures with fresh medium. Column 1 represents parental cells, serially diluted from 1×10^6 to 0.125×10^6 cells, column 2

Fig. 3. Restriction-enzyme analysis of genomic DNA isolated from the parental L1210 cells and from the DFMO-resistant variant cells (L1210/10DC and L1210/10D)

High- M_r DNA (10 μ g) was digested with *HpaII* or *MspI* restriction endonucleases, size-fractionated by electrophoresis and hybridized with nick-translated pODC16. The molecular size markers are shown in the middle.

represents cells continuously treated with DFMO and cadaverine, serially diluted from 0.25×10^6 to 0.03×10^6 cells, and column 3 DFMO-cadaverine-treated cells at 6 weeks after the removal of the diamine, serially diluted from 0.125×10^6 to 0.016×10^6 cells. Rough calculations indicated that the leukaemia cells continuously exposed to DFMO and cadaverine accumulated ODC mRNA in amounts that were 30–50 times higher than those found in the parental cells. However, the omission of cadaverine led to a 300–500-fold overproduction of ODC-specific mRNA in comparison with the parental cells (Fig. 5, column 3).

The polyamine contents of the different cell lines are presented in Table 2. In comparison with the parental cells, the cells treated continuously with DFMO and cadaverine (L1210/10DC) contained only 26% of the natural polyamines (putrescine, spermidine and spermine), which were almost totally replaced by cadaverine-based amines, mainly cadaverine and aminopropyl-

Fig. 4. Amounts of ODC mRNA in untreated L1210 cells (lane 1), in cells continuously exposed to DFMO and cadaverine (lane 2) and in cells from which cadaverine was omitted 6 weeks before the analysis (lane 3)

RNA (10 µg) was size-fractionated by electrophoresis and hybridized with nick-translated pODC16.

cadaverine, as only traces of bis(aminopropyl)cadaverine were found. Upon removal of cadaverine (L1210/10D) the polyamine pattern was completely changed; cadaverine-based amines disappeared and the content of the natural polyamines now represented close to 70 % of that found in the parental cells (Table 2).

DISCUSSION

The present results have connections with two biochemical phenomena that may be of general importance. The first is related to the specificity of polyamine requirement for cellular proliferation, and the second to the mechanisms of gene amplification in general.

Fig. 5. Cyto-dot analysis of the amount of ODC mRNA in untreated L1210 cells (column 1), in cells continuously exposed to DFMO and cadaverine (column 2) and in cells from which cadaverine was removed 6 weeks before the analysis (column 3)

The numbers of cells ($\times 10^6$) applied on to the mRNA affinity paper were as follows (from top to bottom): column 1, 1.0, 0.5, 0.25 and 0.125; column 2, 0.25, 0.125, 0.063 and 0.031; column 3, 0.125, 0.063, 0.031 and 0.016. The two time points (4 and 24 h) refer to the time elapsed from the dilution of the cultures with fresh medium.

As to the specificity of polyamine requirements for cell growth, the present results, together with earlier experimental findings (Alhonen-Hongisto *et al.*, 1982, 1985b) suggest that, although the natural polyamines can be replaced to a large extent by cadaverine-based polyamines, in terms of supporting animal cell growth a certain minimum production of natural polyamines appears to be required. This is exemplified by the fact that, although the total content of cadaverine was fully comparable with that of the natural polyamines in the parental cells (Table 2), these cells overproduced ODC. This overproduction was probably responsible for a continuous formation of spermine, the content of which remained at a value corresponding to more than 50 % of that in the parental cells (Table 2). Interestingly, the overproduction of ODC was almost exclusively based on an enhanced (30–50-fold) accumulation of the ODC mRNA, with practically no changes in the gene dosage for the enzyme. Moreover, the situation appeared to be stable, as the cells exposed to DFMO in the presence of cadaverine continued to overproduce ODC exactly in this fashion for more than 2 years (see Alhonen-Hongisto *et al.*, 1985b). However, although the gene dosage of ODC in L1210/10DC cells was unaltered, the genomic

Table 2. Polyamine concentrations in untreated L1210 leukaemia cells (L1210), in cells continuously exposed to DFMO and cadaverine (L1210/DC) and in cells from which cadaverine was removed 6 weeks before the experiment (L1210/10D)

Results (fmol/cell) are means $\pm$ S.D. for triplicate cultures.

Cell line	Putrescine	Cadaverine	Spermidine	Aminopropylcadaverine	Spermine
L1210	1.21 $\pm$ 0.21	—	3.71 $\pm$ 0.49	—	1.88 $\pm$ 0.23
L1210/10DC	0.22 $\pm$ 0.11	2.30 $\pm$ 0.41	0.46 $\pm$ 0.06	1.54 $\pm$ 0.10	1.09 $\pm$ 0.08
L1210/10D	0.42 $\pm$ 0.04	0.11 $\pm$ 0.01	2.16 $\pm$ 0.28	—	1.78 $\pm$ 0.24

sequences of the enzyme were distinctly hypomethylated in comparison with the parental cells (Fig. 3). The removal of cadaverine, however, resulted in profound changes in the polyamine formation. The overproduction of ODC was dramatically enhanced, owing to an amplification of genomic ODC sequences leading to an enormous accumulation of ODC mRNA. The cellular polyamine pattern was almost normalized, now representing about 65% of that found in the parental cells, with a disappearance of cadaverine-based amines. There are two apparent explanations for the rapid ODC gene amplification upon the removal of cadaverine. Firstly, cadaverine and its aminopropyl derivatives may act as regulatory molecules, preventing gene amplification but allowing an overexpression of ODC. Secondly, the 30–50-fold increase in the production of ODC message may just be the maximum transcriptional activity for a given gene copy number, and to meet the new demands upon the disappearance of the cadaverine-based amines, gene amplification was the only means to increase further the production of ODC.

The present results may also shed some light on the general mechanisms of gene amplification. It is tempting to assume that an amplification of an active gene is in fact preceded by an enhanced transcriptional activity, as proposed by Schimke (1984), possibly associated with gene hypomethylation. The mechanism of the enhanced accumulation of the ODC mRNA in the presence of DFMO and cadaverine may simply be the inability of cadaverine and its aminopropyl derivatives, in contrast with the natural polyamines, to act as 'transcriptional repressors' for ODC. It has to be admitted, however, that, upon removal of cadaverine, the cells experienced a growth crisis, with a substantial decrease in their proliferation rate, lasting for a few weeks. The latter phenomenon may also be causally related to the development of the gene amplification, as impaired DNA synthesis appears to be one of the prerequisites for DNA sequence amplification to occur (Schimke, 1984).

In any event, the present model may be more widely applicable for studies directed to the elucidation of the mechanisms of gene amplification.

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