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A novel mechanism of resistance to α-difluoromethylornithine induced by cycloheximide

Growth with abnormally low levels of putrescine and spermidine

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Treatment of the chemically transformed fibroblasts BP-A31 and other cell lines with low concentrations of cycloheximide (CHM) for 72 h followed by the removal of the protein synthesis inhibitor leads to the proliferation of α-difluoromethylornithine (DFMO)-resistant phenotypes. These drug-resistant cells contain almost no ornithine decarboxylase (ODC) activity and concomitantly very low levels of putrescine and spermidine. Southern blot analysis and measurements of ODC activity and intracellular polyamine levels showed that the described mechanism of inducing resistance to DFMO triggered by CHM does not involve ODC gene amplification, altered transport of the drug or reduced affinity of the enzyme for DFMO.

Cycloheximide *α-Difluoromethylornithine resistance* *Ornithine decarboxylase* *Polyamine*

1. INTRODUCTION

Polyamines have been widely described as playing an important role in cell proliferation and differentiation [1,2]. The ornithine analogue DFMO is an irreversible inhibitor of ODC, the key enzyme in the polyamine biosynthetic pathway. This drug has been extensively used to study the effect of ODC inhibition and reduction of intracellular levels of polyamines on cell proliferation [3]. Chemically transformed mouse fibroblasts such as BP-A31 and other cell lines become arrested when intracellular polyamine levels are markedly reduced after several days of DFMO addition to the tissue culture medium. These cells do not resume growth upon drug removal and can be rescued to proliferate by the addition of exogenous polyamines [4].

Several authors have shown that upon long-term treatment of various cell types with increasing concentrations of DFMO, drug-resistant cells are obtained. The most common mechanism involved is that of ODC gene amplification, with the consequent overproduction of ODC enzyme [5–7]. This mechanism has also been described for several other drugs affecting a variety of different enzymes [8]. Gene amplification of DHFR has been shown to occur in MTX-resistant cells following a multistep procedure of increasing drug concentration, whereas one-step selection with relatively high concentration gives rise to resistant cells showing mutations in either the enzyme (low drug

Dedicated to Dr Luis F. Leloir on the occasion of his 80th birthday

Abbreviations: CHM, cycloheximide; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; DHFR, dihydrofolate reductase; MTX, methotrexate

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affinity) or MTX transport mechanism [9,10]. Mariani and Shimke have demonstrated that amplification of the DHFR gene can also occur in a single cell cycle if synchronized cells are treated with HO-urea (a reversible inhibitor of DNA replication) for 6 h immediately after the beginning of the S phase [11].

This paper describes that treatment of cultures of various cell lines with low concentrations of CHM provokes the proliferation of DFMO-resistant cells upon removal of the protein synthesis inhibitor. ODC enzymatic activity, intracellular polyamine pools and ODC gene pattern of the drug-resistant cells have also been studied.

2. MATERIALS AND METHODS

2.1. *Chemicals and cell lines*

DFMO was a generous gift from Merrell Dow Pharmaceuticals. CHM was purchased from Sigma.

Fibroblasts 3T3-A31, benzo[*a*]pyrene-transformed 3T3 fibroblasts BP-3T3 C17-5 (BP-A31), the human T lymphoid cells YHHH and the human mammary tumor cells MCF-7 were obtained from frozen stocks in this laboratory. The restriction enzyme *Eco*RI was from Bio-Labs and the nitrocellulose paper from Schleicher and Schuell BA-85.

2.2. *DFMO-resistant cell culture (BP-CD5)*

BP-A31 cells were plated at 3.7×10^3 per cm^2 in 35 mm culture petri dishes and grown as in [4]. After 24 h they were shifted to fresh medium containing 0.2 μg/ml of CHM and incubated for 3 days. Cells were then washed twice with PBS and shifted to fresh medium with 5 mM DFMO which was replaced every 3 days.

2.3. *Assay of ODC activity*

Cells were washed quickly with cold PBS, immediately scraped off with a rubber policeman, resuspended in 1 ml of 5 mM Tris-HCl buffer, pH 7.5, containing 2 mM DTT (buffer A) and lysed by freeze-thawing several times after 12 strokes in a Dounce homogenizer. Cell extracts were centrifuged at 100000 $\times$ *g* for 1 h and the supernatant liquids were dialysed against buffer A and then used for ODC activity determinations essentially as described by Insel and Fenno [12]. Protein concentrations were measured by the method of Lowry et al. [13].

2.4. *Determination of intracellular polyamine concentrations*

Cell extracts prepared as described above were treated with 0.2 M perchloric acid and the supernatant fluids obtained after centrifugation for 15 min at 10000 $\times$ *g* were used for the measurement of polyamines by dansylation as in [4].

2.5. *Analysis of genomic DNA*

High-M_r DNA was prepared from BP-A31 and BP-CD5 cells. For the Southern experiments 10 μg DNA was digested with a 5-fold excess of *Eco*RI. The resulting fragments were fractionated by 0.8% agarose gel electrophoresis, transferred onto nitrocellulose and hybridized as described [14] to the nick-translated mouse ODC-cDNA clone pODC54, kindly provided by Dr O.A. Jänne [15]. Filters were subjected to autoradiography for 48–72 h.

3. RESULTS AND DISCUSSION

Working with the transformed cell line BP-A31, we have found that when CHM is added to the culture medium at a concentration able to extend the entire cell cycle more than 3 times, cells resistant to high concentrations of DFMO (5–10 mM) can be obtained immediately after CHM removal (fig.1). The growth in the presence of the drug is observed over the entire cell population with no cellular death. In contrast, the omission of the CHM treatment previous to DFMO addition causes growth inhibition and cellular death after 8–10 days of exposure to the drug at concentrations as low as 0.5 mM. The DFMO-resistant cells (BP-CD5) once selected after the CHM treatment can grow continuously for many months in the presence of 5–20 mM DFMO with a viability as high as that of the parental cells. The growth rate of BP-CD5 cells regardless of the presence of DFMO is lower than that of the wild-type BP-A31 cells (fig.1B).

Table 1 shows a comparative study of the intracellular levels of ODC activity and polyamines in the wild-type and DFMO-resistant cells. BP-CD5 cells contain about 100-times less ODC activity than BP-A31 cells. This extremely low level of

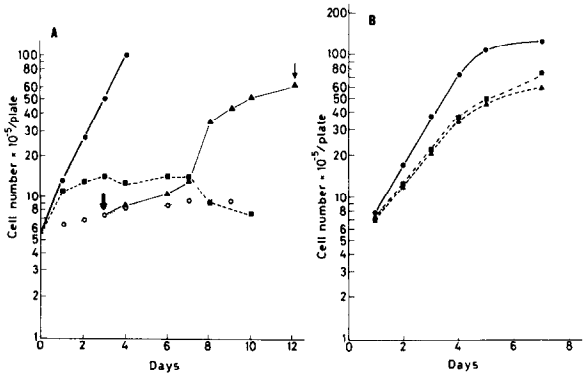

Fig.1. (A) Production of the DFMO-resistant cells BP-CD5. BP-A31 cells were plated as described in section 2. After 24 h they were shifted to the following media: (●) control medium, (■) control medium + 5 mM DFMO, (○) control medium + 0.2 μg/ml CHM, (▲) control medium + 0.2 μg/ml CHM for 3 days, washed twice with PBS and shifted to 5 mM DFMO at the time indicated by the thick arrow. The thin arrow shows the day when the DFMO-resistant cells were trypsinized and replated as the resistant BP-CD5 cells. (B) Growth curves of BP-A31 and BP-CD5 cells. (●) Wild-type BP-A31, (▲) BP-CD5 growing with 5 mM DFMO, (■) BP-CD5 growing in the absence of DFMO.

ODC is reflected in the decreased concentrations of polyamines, particularly those of putrescine and spermidine with minor changes in the spermine content, an observation also described by others [16]. Similar results on the levels of enzyme activity and polyamines were obtained when DFMO was removed 2 days before the preparation of the crude extracts (not shown). In addition, we could not detect cadaverine in the wild-type or DFMO-resistant cells.

Since the drug-resistant cells still have a rather high content of spermine, it is important to emphasize that BP-A31 cells treated with DFMO alone stopped their growth after several days in spite of the fact that spermine levels only decreased to about one-half of that in the untreated cells [4]. Similar observations were reported for several other cell lines [17–19]. The drug-arrested cells can only be rescued to grow by supplementation of culture medium with putrescine [4]. The results shown in table 1 rule out the possibility that BP-CD5 cells have an altered DFMO transport or a DFMO-insensitive ODC enzyme as has been described with other cell lines rendered resistant to DFMO [7,20].

Since ODC gene amplification is a rather common result of drug resistance, we performed a Southern blot analysis of the DNAs derived from the parental and variant cells using a mouse ODC cDNA clone as a probe [15]. Fig.2 shows that the normal pattern of the multigene ODC family [6,21,22] is equally distributed in the wild-type and mutant BP-CD5 cells without any relative increase in the intensity of the different bands corresponding to the resistant cells. This observation is a clear indication that no ODC gene amplification has occurred. Moreover, we have not detected differences in the ODC-mRNA levels between mutant and wild-type cells (not shown).

The present results are not exclusive to the mouse fibroblast cell line BP-A31. DFMO-resistant cells can also be obtained using the same CHM/DFMO treatment with the normal

Table 1

Ornithine decarboxylase activity and intracellular polyamine pools of BP-A31 and BP-CD5 cells

Cells	ODC activity (nmol/h per mg protein)	Polyamines (nmol/mg protein)		
		Putrescine	Spermidine	Spermine
BP-A31	3.62	6.54	16.88	5.58
BP-CD5	0.04	0.38	0.86	3.09

ODC activity values given in nmol $^{14}CO_2$ released from L-[1-^{14}C]ornithine were corrected for radioactivity in control experiments carried out without enzyme

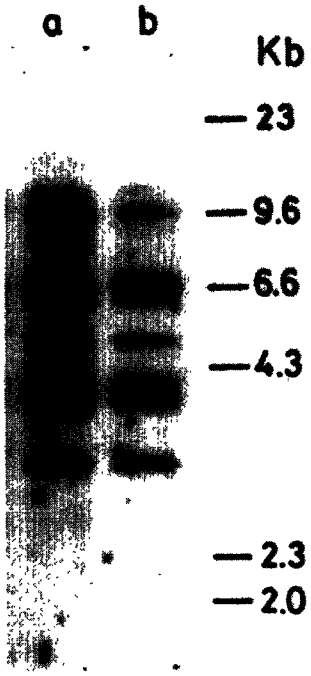

Fig.2. Analysis of genomic DNA obtained from wild-type BP-A31 (a) and DFMO-resistant BP-CD5 cells (b). Each lane contains 10 μg *Eco*RI-digested DNA. Other details as in section 2.

fibroblast 3T3-A31 as well as with the human T lymphoid cell line YHHH [14] and the human mammary tumor cell line MCF-7 (not shown).

The above-mentioned observations seem to indicate that putrescine and spermidine are no longer essential for cellular growth if cells have been previously treated with low doses of the protein synthesis inhibitor CHM. It is known that this drug can produce several genetic and epigenetic modifications like double replication of chromosomal DNA segments [23], superinduction of proteins [24,25], superinduction of c-myc mRNA [26,27] and a transient reversion of the tumoral phenotype in viral and chemically transformed cells [28]. The mechanism by which DFMO-resistant cells were produced in our case does not involve gene amplification, the appearance of a DFMO-insensitive ODC, nor a decreased transport of DFMO. It seems that CHM does not cause damage (at least permanent) to the ODC gene or its product, since 6–8 days after DFMO removal, the cells show increasing ODC activity with the consequent rise in the levels of polyamines. These results suggest that CHM probably acts by inducing the expression of some other gene(s), which allows the cell to proliferate with very low levels of putrescine and spermidine. To our knowledge, this is a novel mechanism of resistance to DFMO induced by CHM. Other inhibitors of protein synthesis like histidinol and puromycin also used at suboptimal concentrations did not produce the effect obtained with CHM (not shown).

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