Characterization of difluoromethylornithine-resistant mouse and human tumour cell lines

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Four mouse and two human tumour cell lines resistant to α -difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), were analysed for the activities of polyamine-biosynthetic and -biodegradative enzymes as well as for cellular polyamine contents. In all but one of these cell lines the resistance to DFMO was based on an overproduction of ODC. In a human myeloma cell line the resistance was based on a greatly enhanced arginase activity. Except for one L1210 variant cell line, all the resistant cell lines contained elevated *S*-adenosylmethionine decarboxylase activity. Similarly, all the resistant mouse, but not human, cell lines displayed enhanced spermidine and spermine synthase activities. Arginase activity was detected only in human cell lines. In both DFMO-resistant cell lines the activity of arginase was strikingly elevated. Of the biodegradative enzymes, polyamine oxidase activity was readily detectable in all mouse cells, but no measurable activity was found in the human cells. Spermidine/spermine *N*¹-acetyltransferase activity was elevated in three out of four resistant mouse cell lines. Even though the concentration of spermidine was usually lower in the overproducer cells, this was compensated by an increased content of spermine. The two resistant human myeloma cells contained intracellular ornithine concentrations that were from more than 5 to more than 20 times higher than those in the parental cells.

INTRODUCTION

Mouse and human tumour cells rapidly acquire resistance to DFMO, an anti-proliferative agent that acts through an irreversible inhibition of ODC. Overproduction of ODC based on an amplification of transcriptionally active genomic sequences is a well-documented mechanism for the development of resistance (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985b; Leinonen *et al.*, 1987). The overproduction of ODC can occur also in the absence of an apparent gene amplification through enhanced transcription (Alhonen-Hongisto *et al.*, 1985a; McConlogue *et al.*, 1986) or even through an improved translational efficiency of normal amounts of mRNA (McConlogue *et al.*, 1986).

In contrast with these ODC-overproducer cells, we recently selected a DFMO-resistant human myeloma cell line in which the resistance was based on a greatly elevated arginase activity without any changes in the gene dosage or expression of ODC (Alhonen-Hongisto *et al.*, 1987b). In these cells, the high arginase activity probably produced ornithine in amounts sufficient to compete intracellularly with DFMO and hence prevent the inactivation of ODC by the drug (Alhonen-Hongisto *et al.*, 1987b).

We have now collected all our DFMO-resistant mouse and human tumour cell variants and made a thorough comparison between these cells and their respective parental cells. We have measured all the biosynthetic and biodegradative enzymes of the polyamines as well as the actual polyamine pools of the cells. Several interesting

features have emerged. Firstly, in human cells arginase appears to have a special role in the development of resistance to DFMO, as, in addition to the previously described human myeloma (Alhonen-Hongisto *et al.*, 1987b), another DFMO-resistant human myeloma cell line (Sultan) with marked ODC-gene amplification displayed nearly 20-fold increase in arginase activity. Secondly, polyamine oxidase, which in mouse cell lines displayed rather high activity and was apparently constitutively expressed, was undetectable in all four human cell lines. Thirdly, the overexpression of ODC in all cell lines was sufficient to maintain normal total polyamine pools, in spite of the presence of up to 20 mM-DFMO.

EXPERIMENTAL

Cell lines and culture conditions

The cell lines used in this study are listed in Table 1, with short description and the reference of origin whenever applicable. The extension such as /10D refers to the concentration (10 mM) of DFMO (D) to which the cells are resistant. The letter C refers to cadaverine (5 μ M).

All the cells were grown in suspension cultures in RPMI 1640 medium (Gibco, Paisley, Scotland, U.K.) supplemented with 5% (v/v) pooled human serum (Finnish Red Cross Transfusion Service, Helsinki, Finland), antibiotics (penicillin and streptomycin) and, for the resistant cells, an appropriate concentration of DFMO (see Table 1). The cells were harvested for biochemical analyses at 24 h after dilution of the cultures with fresh medium containing DFMO (and cadaverine).

Abbreviations used: DFMO, α -difluoromethylornithine; ODC, ornithine decarboxylase (EC 4.1.1.17); AdoMetDC, *S*-adenosylmethionine decarboxylase (EC 4.1.1.50).

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Table 1. DFMO-resistant mouse and human tumour cell lines

Cell line	Description	Reference
Mouse		
L1210	Mouse leukemia; parental cell line	—
L1210/10DC	Grown for several years in the presence of 10 mM-DFMO and 5 μ M-cadaverine. Overproduces ODC and its mRNA; gene copy number unchanged; hypomethylated sequences	Alhonen-Hongisto <i>et al.</i> (1985a)
L1210/10D	Derived from L1210/10DC upon omission of cadaverine. Overproduces ODC owing to gene amplification	Alhonen-Hongisto <i>et al.</i> (1987a)
EAC	Ehrlich ascites carcinoma; parental cell line	—
EAC/20D	Overproduces ODC owing to gene amplification	Alhonen-Hongisto <i>et al.</i> (1985b)
X63	Mouse myeloma; parental cell line	—
X63/20D	Overproduces ODC owing to gene amplification	—
Human		
Sultan	Human myeloma (IgG); parental cell line	Ralph (1979)
Sultan/20D	Overproduces ODC owing to gene amplification	Leinonen <i>et al.</i> (1987)
Fravel	Human myeloma; parental cell line	Miller <i>et al.</i> (1982)
Fravel/4D	Resistant to 4 mM-DFMO; no overproduction of ODC; enhanced arginase activity	Alhonen-Hongisto <i>et al.</i> (1987b)

Materials

DFMO was generously given by Centre de Recherche Merrell International (Strasbourg, France). DL-[1-¹⁴C]-Ornithine (sp. radioactivity 61 Ci/mol), DL-[U-¹⁴C]-arginine (sp. radioactivity 300 Ci/mol), DL-[1-¹⁴C]-methionine (sp. radioactivity 50 Ci/mol), [¹⁴C]-spermine (sp. radioactivity 100 Ci/mol), 5'-[α -³²P]-dCTP (sp. radioactivity 410 Ci/mmol) and [1-¹⁴C]-acetyl-CoA (sp. radioactivity 54 Ci/mol) were purchased from Amersham International (Amersham, Bucks., U.K.). Radioactive S-adenosyl-L-methionine was prepared enzymically by the method of Pegg & Williams-Ashman (1969). Decarboxylated adenosyl-methionine was prepared by the method of Pösö *et al.* (1976) and further purified by Cellex-P chromatography (Eloranta *et al.*, 1976).

Analytical methods

The activities of ODC and AdoMetDC were assayed by the methods of Jänne & Williams-Ashman (1971a,b). The activity of arginase was determined by the method of Alhonen-Hongisto *et al.* (1982). Stoichiometric formation of radioactive urea and ornithine from uniformly labelled arginine was confirmed by using an amino acid analyser. The activity of polyamine oxidase was assayed by the method of Hölttä (1977). The activities of spermidine and spermine synthases were assayed as described by Raina *et al.* (1983). The activity of spermidine/spermine N¹-acetyltransferase was determined by the method of Matsui *et al.* (1981).

Polyamine and ornithine concentrations were determined by a LKB Alpha Plus amino acid analyser essentially as described previously (Mach *et al.*, 1981; Hyvönen *et al.*, 1988).

Genomic DNA was isolated by the method of Blin & Stafford (1976). DNA was digested with *Msp*I (Amersham International) restriction endonuclease, electrophoresed in 0.9%-agarose gels, transferred to nitrocellulose filters (Southern, 1975) and hybridized to nick-translated (Rigby *et al.*, 1977) pODC10/2H, a human ODC cDNA clone (kindly provided by Dr. O. A. Jänne).

The significance of the differences was computed by Student's two-tailed *t* test.

RESULTS

The activities of five biosynthetic enzymes of the polyamines in 11 mouse and human tumour cell lines are depicted in Table 2. An interesting difference between the two species becomes apparent at the first sight. Although all the human cell lines contained high arginase activity, in comparison with rest of the biosynthetic enzymes, this activity was undetectable in all but one of the mouse cell lines. Arginase activity was enhanced 17-fold in the two human DFMO-resistant variants (Sultan/20D and Fravel/4D). Although undetectable in mouse myeloma cells (X/63), the DFMO-resistant mouse myeloma cells (X63/20D) exhibited arginase activity which was comparable with that found in the human parental myeloma cells (Sultan and Fravel) (Table 2). However, repeated determinations (at 12, 24 and 48 h) of arginase activity indicated that the enzyme was found only occasionally (owing to low basal activity) in these mouse cells. Except for Fravel/4D cells, all the DFMO-resistant cells displayed a strikingly elevated ODC activity (Table 2). Repeated assays of arginase activity at 12, 24 and 48 h after the addition with fresh medium revealed little cell-cycle-specific changes of the enzyme activity, which

Table 2. Biosynthetic enzymes for the polyamines in parental and DFMO-resistant mouse and human tumour cell lines

The activities (pmol/10⁶ cells per 60 min) are means $\pm$ S.D. for triplicate cultures (n.d., not detected): **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Cell line	Arginase	ODC	AdoMetDC	Spermidine synthase	Spermine synthase
Mouse					
L1210	n.d.	30 $\pm$ 5	88 $\pm$ 16	2230 $\pm$ 310	237 $\pm$ 25
L1210/10DC	n.d.	369 $\pm$ 38***	1650 $\pm$ 201***	2820 $\pm$ 201*	282 $\pm$ 45
L1210/10D	n.d.	1680 $\pm$ 126***	66 $\pm$ 3	2760 $\pm$ 262	364 $\pm$ 17***
EAC	n.d.	76 $\pm$ 5	31 $\pm$ 1	2170 $\pm$ 182	343 $\pm$ 26
EAC/20D	n.d.	1130 $\pm$ 97***	243 $\pm$ 16***	2630 $\pm$ 297	460 $\pm$ 24**
X63	n.d.	49 $\pm$ 20	36 $\pm$ 8	2050 $\pm$ 109	276 $\pm$ 64
X63/20D	4710 $\pm$ 1880	1170 $\pm$ 77***	72 $\pm$ 10**	2710 $\pm$ 406*	316 $\pm$ 32
Human					
Sultan	3930 $\pm$ 480	32 $\pm$ 3	40 $\pm$ 3	1970 $\pm$ 228	414 $\pm$ 7
Sultan/20D	64900 $\pm$ 5150***	743 $\pm$ 52***	85 $\pm$ 2***	1620 $\pm$ 353	367 $\pm$ 27*
Fravel	8020 $\pm$ 760	29 $\pm$ 5	50 $\pm$ 6	2020 $\pm$ 132	565 $\pm$ 63
Fravel/4D	137000 $\pm$ 3610***	10 $\pm$ 0.5**	467 $\pm$ 11***	1113 $\pm$ 154***	98 $\pm$ 7***

remained strikingly elevated in the resistant cells. As we now for the first time observed that not only DFMO-resistant Fravel cells but also resistant Sultan cells contained elevated arginase activity, we wondered whether the nature of resistance of the Sultan cells has been changed since our previous report (Leinonen *et al.*, 1987). As depicted in Fig. 1, Southern-blot analysis of genomic DNA obtained from Sultan/20D cells showed that cells still contained highly amplified genomic ODC sequences. Similar analysis of genomic DNA obtained from Fravel and Fravel/4D cells revealed no differences of ODC gene dosage (results not shown).

Except for one cell line (L1210/10D), all the resistant cell lines contained grossly elevated AdoMetDC activity. The stimulation did not change when the enzyme assays were repeated at 12, 24 and 48 h, except for X63/20D cells, in which no significant difference was found at 12 h (92 $\pm$ 18 versus 108 $\pm$ 39). Relatively small changes were found in the activities of the two propylamine transferases. Both activities were increased in the resistant mouse, but not human, cell lines. In fact, the most profound changes in spermidine and spermine synthases occurred in DFMO-resistant Fravel cells (Fravel/4D), in which the activity of spermidine synthase was decreased by 50% and that of spermine synthase by more than 80% (Table 2).

We next screened all the cell lines for enzyme activities involved in the catabolism (or backward conversion) of the polyamines, namely spermidine/spermine *N*¹-acetyltransferase and polyamine oxidase. Again, an interesting difference between the two species (mouse and man) emerged. The activity of polyamine oxidase, which was rather high in all the mouse cell lines, was undetectable in the two human cell lines (Table 3). When the assays of polyamine oxidase activity were repeated at 12, 24 and 48 h, the human cell lines did not exhibit any detectable activity at any of the time points. In mouse cells the enzyme activity appeared to be constitutively expressed, as the time after dilution with fresh medium had practically no effect on the enzyme activity. Although DFMO-resistant L1210 and Ehrlich ascites-carcinoma cells contained elevated acetyltransferase activity (Table 3), there were no general signs indicating that the back-

Fig. 1. Restriction-enzyme (*Msp*I) analysis of ODC sequences in genomic DNA isolated from parental or DFMO-resistant human myeloma cells

DNA isolated from parental Sultan cells (lane C) or from Sultan/20D cells (lane D) was digested with *Msp*I restriction endonuclease, size-fractionated by electrophoresis and hybridized with nick-translated pODC10/2H. The molecular-size markers are shown to the right.

conversion of polyamines would have been activated in the DFMO-resistant cells (Table 3).

Intracellular polyamine pools in all the cell lines are listed in Table 4. In general, one may notice that an overproduction of ODC and the accompanying changes

in other enzyme activities (most notably AdoMetDC) can fully maintain normal pools of total polyamines in most of the resistant cell lines. Admittedly, the polyamine pattern was 'biased' towards spermine, i.e. the proportion of spermine was much higher in the resistant cells. L1210/10DC and Fravel/4D cells represented a distinct exception to this general rule. In L1210/10DC cells the natural polyamines were to a large extent replaced by cadaverine-based amines as they were exposed to DFMO in the presence of cadaverine. In Fravel/4D cells the total polyamine pool was decreased by 50 %, which still appears to support near-normal cellular proliferation. Measurable concentrations of *N*¹-acetylspermidine were found in some cell lines and, apart from the L1210/10DC cells, traces of cadaverine were found occasionally (Table 4).

Table 3. Spermidine/spermine *N*¹-acetyltransferase and polyamine oxidase activities in parental and DFMO-resistant mouse and human tumour cell lines

The activities (pmol/10⁶ cells per 60 min) are means $\pm$ s.d. for triplicate cultures (n.d., not detected): **P* < 0.05; ***P* < 0.01.

Cell line	Spermidine/spermine <i>N</i> ¹ -acetyltransferase	Polyamine oxidase
Mouse		
L1210	75 $\pm$ 19	592 $\pm$ 63
L1210/10DC	105 $\pm$ 20	402 $\pm$ 59*
L1210/10D	141 $\pm$ 1**	441 $\pm$ 10**
EAC	105 $\pm$ 25	382 $\pm$ 41
EAC/20D	185 $\pm$ 15**	303 $\pm$ 11*
X63	91 $\pm$ 12	405 $\pm$ 33
X63/20D	90 $\pm$ 13	319 $\pm$ 37*
Human		
Sultan	88 $\pm$ 21	n.d.
Sultan/20D	67 $\pm$ 4	n.d.
Fravel	109 $\pm$ 45	n.d.
Fravel/4D	79 $\pm$ 11	n.d.

The results presented in Table 5 indicate that the enhanced arginase activity in the resistant human cells (Sultan/20D and Fravel/4D) resulted in substantial increases in intracellular ornithine pools. In the resistant Sultan cells (Sultan/20D) ornithine concentration was 5-fold and in the Fravel/4D cells 20-fold higher than that in their respective parental cells (Table 5). In fact, the concentration of intracellular ornithine in the Fravel/4D cells was higher than that of intracellular DFMO (results not shown).

DISCUSSION

The purpose of the present study was to elucidate possible changes in polyamine-biosynthetic enzymes after development of resistance to DFMO, the irreversible inhibitor of ODC. Of six DFMO-resistant mouse and human cell lines, five exhibited distinctly elevated AdoMetDC activity (Table 2). It thus appears that an enhanced decarboxylation of *S*-adenosylmethionine is closely associated with the overproduction of ODC in DFMO-resistant tumour cells. On the other hand, no consistent changes were found in the activities of the two

Table 5. Intracellular concentration of ornithine in parental and DFMO-resistant human myeloma cells

Results (pmol/10⁶ cells) are means $\pm$ s.d. for triplicate cultures.

Cell line	Ornithine concn.
Sultan	< 80
Sultan/20D	390 $\pm$ 170
Fravel	< 70
Fravel/4D	1440 $\pm$ 230

Table 4. Polyamine concentrations in parental and DFMO-resistant mouse and human tumour cell lines

The results (pmol/10⁶ cells) are means $\pm$ s.d. for triplicate cultures [n.d., not detected; traces, found in some samples (contributes to the total polyamines)]: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Cell line	Putrescine	Spermidine	Spermine	<i>N</i> ¹ -Acetyl-spermidine	Cadaverine	Aminopropyl-cadaverine	Total
Mouse							
L1210	510 $\pm$ 61	1630 $\pm$ 94	874 $\pm$ 10	n.d.	traces	traces	3140 $\pm$ 68
L1210/10DC	n.d.	53 $\pm$ 7***	705 $\pm$ 44**	n.d.	2720 $\pm$ 253	1250 $\pm$ 148	4730 $\pm$ 446*
L1210/10D	323 $\pm$ 30**	1610 $\pm$ 139	1460 $\pm$ 130***	n.d.	n.d.	n.d.	3390 $\pm$ 270
EAC	969 $\pm$ 98	2920 $\pm$ 312	1430 $\pm$ 275	80 $\pm$ 12	n.d.	n.d.	5400 $\pm$ 604
EAC/20D	262 $\pm$ 55***	1400 $\pm$ 92***	2860 $\pm$ 82***	38 $\pm$ 15	traces	traces	4600 $\pm$ 211
X63	1180 $\pm$ 44	2160 $\pm$ 178	942 $\pm$ 75	n.d.	n.d.	n.d.	4280 $\pm$ 264
X63/20D	664 $\pm$ 114***	2430 $\pm$ 450	1590 $\pm$ 296*	n.d.	n.d.	n.d.	4680 $\pm$ 851
Human							
Sultan	542 $\pm$ 2	1400 $\pm$ 43	1650 $\pm$ 132	25 $\pm$ 5	n.d.	n.d.	3620 $\pm$ 167
Sultan/20D	280 $\pm$ 13***	1250 $\pm$ 147	2580 $\pm$ 78***	69 $\pm$ 41	n.d.	n.d.	4170 $\pm$ 34**
Fravel	389 $\pm$ 99	1230 $\pm$ 209	1760 $\pm$ 221	n.d.	n.d.	n.d.	3380 $\pm$ 527
Fravel/4D	traces	175 $\pm$ 26***	1590 $\pm$ 91	n.d.	n.d.	n.d.	1790 $\pm$ 55**

propylamine transferases, even though these activities tended to be higher in resistant mouse cells, but not in human cells. In comparison with the increases in AdoMetDC (or ODC) activities in the DFMO-resistant cells, however, the stimulation of the transferase activities was negligible. The same conclusion also applies to the activities of the biodegradative enzymes, spermidine/spermine *N*¹-acetyltransferase and polyamine oxidase, as no consistent changes were found to be associated with the development of DFMO-resistance (Table 3). In other words, we found little evidence for the activation of the backward reaction, i.e. conversion of spermine ultimately into putrescine.

In most cases, the increases in ODC and AdoMetDC activities were obviously sufficient to maintain normal intracellular polyamine pools even in the presence of up to 20 mM-DFMO.

In the light of the present results, the central role of arginase in the synthesis of the polyamines and in the development of DFMO-resistance is becoming more and more obvious. In addition to the recently described (Alhonen-Hongisto *et al.*, 1987b) human myeloma (Fravel) cell line resistant to DFMO by virtue of strikingly elevated arginase activity, we now found similar stimulation of arginase activity in another human myeloma (Sultan) resistant to DFMO through gene-amplification-based overproduction of ODC. Whether the stimulation (or even the existence) of cellular arginase activity is typical of human cells or of myeloma is not known at present. In any event, both of the human resistant myeloma cell lines also contained very high intracellular ornithine pools, probably at least partly sufficient to overcome the inhibition of ODC by DFMO (Metcalfe *et al.*, 1978). Several pieces of experimental evidence seem to indicate that arginase functions as a polyamine-biosynthetic enzyme in non-ureotelic cells and can be coordinately induced with ODC and AdoMetDC (Klein & Morris, 1978; Oka & Perry, 1974). This view is also supported by the findings of Hölttä & Pohjanpelto (1982), showing that arginase deficiency leads to polyamine dependency. Thus, in the future, arginase may become one of the central targets for cancer chemotherapy based on polyamine deprivation. On the other hand, the significance of polyamine oxidase or the recycling reaction system in general may be of less importance in human tumours than in mouse neoplasms.

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