Human myeloma cells acquire resistance to difluoromethylornithine by amplification of ornithine decarboxylase gene

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Stepwise increments of the concentration of 2-difluoromethylornithine (DFMO), a mechanism-based irreversible inhibitor of mammalian ornithine decarboxylase (ODC), resulted in a selection of cultured human IgG-myeloma cells (Sultan cell line) capable of growing in the presence of up to 3 mM-DFMO. This capacity was associated with (i) 10-fold increase in ODC activity in the dialysed extracts of drug-resistant myeloma cells, (ii) markedly enhanced synthesis rate for ODC enzyme molecules, as revealed by a 20 min [³⁵S]methionine labelling of cellular proteins, followed by specific immunoprecipitation and SDS/polyacrylamide-gel electrophoresis, (iii) dose-dependently increased expression of ODC mRNA in resistant cells (effective dose causing 50% inhibition), (iv) dose-dependent amplification of ODC gene sequences in a 9-kilobase-pairs *Eco*RI genomic DNA fragment, and (v) a 10-fold increase in the ED₅₀ (effective dose causing 50% inhibition) for the anti-proliferative action of DFMO in these myeloma cells. These results represent one of the few gene amplifications described in cultured human cells.

INTRODUCTION

Gene amplification is a well-documented mechanism by which cells become able to meet the demand for increased quantities of specific gene products under toxic or metabolic stress (Stark & Wahl, 1984; Schimke, 1984). In addition to becoming a frequently reported phenomenon in the development of drug-resistant cell variants of mainly lower-mammalian (rodent) origin *in vitro*, the importance of gene amplification in the development of resistance to cancer chemotherapeutic agents in clinical medicine has also gained wider interest (Schimke *et al.*, 1984).

Ornithine decarboxylase (ODC), the rate-controlling enzyme of polyamine biosynthesis, is one of the few enzymes the genes for which undergo amplification under appropriate selection pressure (Schimke, 1984). Several mouse cell lines have been shown to amplify their ODC genes on exposure to 2-difluoromethylornithine (DFMO) (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985a,c), a mechanism-based irreversible inhibitor of the enzyme (Metcalf *et al.*, 1978), for long periods of time.

We have exposed cultured human myeloma cells to increasing concentrations of DFMO resulting in a selection of tumour cells resistant to 3 mM concentrations of DFMO. The genomic organization of ODC genes (restriction-enzyme pattern) in human myeloma cells is similar to that of fresh leucocytes obtained from leukaemia patients, in whom favourable clinical response to DFMO has been documented (Siimes *et al.*, 1981), but markedly different from the restriction-enzyme pattern of mouse cells (Alhonen-Hongisto *et al.*, 1985a,c). Under exposure to DFMO these human cells appear to undergo a dose-dependent amplification of one of their ODC genes, resulting in increased synthesis and activity of ODC. Together with the findings indicating that cells derived from tumours of patients treated with metho-

trexate have increased dihydrofolate reductase gene copy number (Curt *et al.*, 1983; Horns *et al.*, 1984; Trent *et al.*, 1984; Carman *et al.*, 1984), our results may have potential implications in the development of cancer chemotherapeutic regimens. In fact, this is one of the very few examples of cultured human cell lines undergoing gene amplification in response to anti-proliferative treatment.

EXPERIMENTAL

Selection of DFMO-resistant cells

Human IgG-myeloma cells (Sultan cell line derived from bone marrow of a patient with IgG myeloma; Goldblum *et al.*, 1973; Ralph, 1979) were grown in suspension cultures in RPMI 1640 medium (Gibco) supplemented with 5% pooled human serum (Finnish Red Cross Transfusion Service, Helsinki, Finland) and antibiotics. The cells were initially grown in the presence of 0.05 mM-DFMO (generously given by Centre de Recherche Merrell International, Strasbourg, France) for about 1 week, whereafter the concentration of DFMO was increased stepwise (→0.1 mM→0.2 mM→0.4 mM→0.6 mM→0.8 mM→1.0 mM→1.5 mM→2.0 mM→3.0 mM) over a period of 6 months (several weeks at each concentration). All the experiments described in this paper, however, were performed with cells adapted to grow in the presence of 1 mM-DFMO.

Analytical methods

ODC activity was assayed by the method of Jänne & Williams-Ashman (1971). Biosynthetic labelling of ODC with [³⁵S]methionine (sp. radioactivity 1095 Ci/mmol) (New England Nuclear Corp., Dreieich, West Germany) was as follows. About 5 × 10⁶ cells were washed and resuspended in 400 µl of methionine-free minimal Eagle's medium (Gibco) with 2% human serum and

Fig. 1. Immunoprecipitation of [^{35}S]methionine-labelled ODC from parental and DFMO-resistant human myeloma cells

Parental and DFMO-adapted (1.0 mM) human myeloma cells (5×10^6 cells) were incubated in the presence of $10 \mu\text{Ci}$ of [^{35}S]methionine for 20 min, whereafter the cytosolic peptides were immunoprecipitated and analysed by SDS/10% polyacrylamide gel electrophoresis as described in the Experimental section. M_r standards were bovine serum albumin (69000), ovalbumin (46000) and carbonic anhydrase (30000).

preincubated for 90 min, whereafter $10 \mu\text{Ci}$ of [^{35}S]methionine was added and cells were incubated at 37°C for 20 min with constant agitation. Cells were then washed twice with 0.5 ml of RPMI 1640 medium, resuspended in $200 \mu\text{l}$ of 150 mM-NaCl/5 mM-EDTA/50 mM-Tris/0.5% Nonidet P-40/0.1% SDS/2 mM-methionine and subjected to three cycles of rapid freezing and thawing. Immunoprecipitation of [^{35}S]methionine-labelled ODC molecules was performed essentially as described by Kraus & Rosenberg (1982) with monospecific anti-ODC antibody (Isomaa *et al.*, 1983). The immunoprecipitated peptides were fractionated by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970). After the electrophoresis, the gels were treated with EN ^{3} HANCE (New England Nuclear Corp.) and fluorography of the dried gels was carried out by exposing them to Kodak XAR film up to 9 days. ^{14}C -labelled M_r marker proteins were from New England Nuclear Corp.

Total cellular RNA was isolated by one method of Auffray & Rougeon (1980) and DNA essentially as described by Blin & Stafford (1976). RNA was fractionated by electrophoresis in the presence of formaldehyde in 1.4% agarose gels, transferred on to Gene-Screen (New England Nuclear Corp.) and hybrid-

Fig. 2. Amounts of ODC mRNA in human myeloma cells exposed to increasing concentrations of DFMO

RNA was extracted from human myeloma cells grown in the presence of DFMO concentrations shown in the Figure for at least 1 month after stepwise increments up to that concentration as described in the Experimental section. The size fractionation of RNA ($10 \mu\text{g}$) on formaldehyde-containing (1.4%) agarose gels was followed by its transfer on to Gene-Screen for hybridization with nick-translated pODC16 probe. Molecular size markers are shown to the right.

ized to nick-translated (Rigby *et al.*, 1977) cDNA probe pODC16, encoding mRNA of mouse kidney ODC (Kontula *et al.*, 1984; Jänne *et al.*, 1984), as described by Thomas (1980). Isolated genomic DNA was digested with *EcoRI* restriction endonuclease (Boehringer, Mannheim, West Germany) (the insert has no internal cleavage site for *EcoRI*), fractionated by electrophoresis in 0.9% agarose gels, transferred on to nitrocellulose filters (Southern, 1975) and hybridized to nick-translated pODC16. The specific radioactivity of the nick-translated pODC16 [^{32}P]dCTP was 0.5×10^8 – 1×10^8 c.p.m./ μg of DNA. pODC16 containing an insert of cDNA of mouse kidney ODC mRNA (mainly coding segment) is a suitable probe for blot-hybridization analyses with DNA or RNA of human origin, as the sequence homology between murine and human ODC mRNA is more than 90% (O. A. Jänne, unpublished work). Experiments in which a human cDNA-containing plasmid (pODC10/2H) (Winqvist *et al.*, 1986) was used as the probe gave a hybridization pattern identical with that obtained with mouse pODC16.

RESULTS

ODC activity, as measured with dialysed extracts to eliminate partly the inhibition of the enzyme activity by free DFMO, was about 10-fold higher in cells adapted to grow in 1.0 mM-DFMO than in control cells. As expected, the differences in the synthetic rates (as determined with short-time biosynthetic labelling with [^{35}S]methionine) were clearly greater (Fig. 1). The apparent M_r of immunoreactive ODC in human myelomas, in both parental and DFMO-adapted cells, was 52000. The band of apparent M_r 42000 seen in Fig. 1 is actin, which is to some extent co-precipitated with anti-ODC antibody, as it co-precipitates with other monospecific antibodies such as anti-fibronectin anti-

Fig. 3. Restriction analysis of genomic sequences of ODC in human myeloma cells exposed to increasing concentrations of DFMO

DNA was extracted from cells digested with *Eco*RI restriction endonuclease, fractionated by electrophoresis (10 μ g in each lane), transferred on to nitrocellulose filters and hybridized to pODC16 probe as detailed in the Experimental section. Molecular size markers are shown to the right.

body used as a non-specific antibody in the present study. In the resistant cells, ODC activity was enhanced 20-fold and its synthesis by more than 30-fold, as revealed by serial dilution before the fluorography (results not shown).

The expression of ODC mRNA in the tumour cells was enhanced dose-dependently under the pressure of different doses of DFMO (Fig. 2). The size of human ODC mRNA was 2.3 kb, which is similar to mouse ODC mRNA (Alhonen-Hongisto *et al.*, 1985a). At low washing stringency (about melting temperature -20°C) pODC16 also appeared to recognize 5-kb transcript sequences. The nature of these sequences is not completely clear at the moment, but the available evidence suggests that there are shared sequences between 28S rRNA and eukaryotic ODC mRNA. This view is based on the following findings: (i) 28S rRNA co-purifies with total RNA; (ii) it is not polyadenylated; and (iii) commercial calf liver 28S rRNA also hybridizes, although weakly, with pODC16. Moreover, there was no apparent signal enhancement associated with the increase in ODC mRNA in response to chronic DFMO exposure (results not shown). Direct sequence comparisons between human 28S rRNA and mouse ODC cDNA are required.

The enhanced expression of ODC in the drug-resistant cells was at least partly based on amplification of its

Fig. 4. Inhibition of the growth of parental and ODC-producing myeloma cells by DFMO

Parental and DFMO-adapted (1.0 mM) human myeloma cells ($0.4 \times 10^6/\text{ml}$) were grown for 72 h in the presence of different concentrations of DFMO, whereafter cell densities were determined.

gene(s). Two *Eco*RI restriction fragments (9 kb and 3 kb) of human genomic DNA hybridized with pODC16, and, in particular, the major 9 kb fragment was dose-dependently amplified in cells exposed to increasing concentrations of DFMO. It is difficult to say whether also the 3 kb fragment (barely visible in Fig. 3) was amplified. The amplification was confirmed by using other restriction enzymes. After cleavage with *Hpa*II, three signals were seen, one of which was amplified (results not shown). The filters were also hybridized with a *N-myc* probe, as the genes of ODC and *N-myc* reside in the same human chromosome (Winqvist *et al.*, 1986), but no change in the gene dosage of *N-myc* was associated with the amplification of ODC sequences (Alhonen-Hongisto *et al.*, 1987). It is noteworthy that the *Eco*RI restriction fragments in these human cells completely differ from those obtained from mouse genome (Alhonen-Hongisto *et al.*, 1985a,c), but are similar to those we have found from genomic DNA of fresh human leukaemic cells (results not shown).

The enhanced expression of the ODC gene in human myeloma cells grown under the pressure of DFMO was also evident in their increased resistance towards the anti-proliferative action of DFMO (Fig. 4). When the cells were grown for 72 h in the presence of DFMO, the ED_{50} (effective dose causing 50% inhibition) was 0.13 mM for the control cells and 1.5 mM for cells adapted to grow in the presence of 1 mM-DFMO.

DISCUSSION

Together with results from other groups (McConlogue *et al.*, 1984; Kahana & Nathans, 1984) our previous studies have substantiated the evidence that ODC is one of about a dozen mammalian enzymes the genes of which easily undergo amplification under appropriate selection pressure (Alhonen-Hongisto *et al.*, 1985a,c). So far,

almost all the induced mammalian gene amplifications have dealt with rodent, mainly mouse, cell lines, many of which are known to be very prone to spontaneous mutations and gene rearrangements (reviewed by Stark & Wahl, 1984; and Schimke, 1984). Since it is feasible that a particular gene may be differently susceptible to amplification in different genomic organizations, i.e. in different species, we have now studied ODC-gene amplification in human cells.

In contrast with mouse cell lines (McConlogue *et al.*, 1984; Kahana & Nathans, 1984; Alhonen-Hongisto *et al.*, 1985a,c), the human myeloma cells appeared to yield a smaller number of restriction fragments that react with ODC cDNA. In fact, marine and human cDNAs for ODC are more than 90% homologous (O. A. Jänne, unpublished work), thus making it possible to use either of them as probes. The *EcoRI* restriction fragment pattern in myeloma cells was similar to those obtained from fresh cells from leukaemia patients (L. Alhonen-Hongisto, P. Leinonen & J. Jänne, unpublished work), suggesting that they are truly representative of human cells (this Sultan cell line has been maintained in culture for more than 10 years). The size of the ODC mRNA (2.3 kb), and the apparent M_r of the immunoprecipitated enzyme (52000) in human cells, are similar to those reported for mouse (Kontula *et al.*, 1984; Alhonen-Hongisto *et al.*, 1985a,c; Isomaa *et al.*, 1983). However, unlike mouse cells, where, owing to differences in utilization of two polyadenylation signals (Hickok *et al.*, 1986), there are two ODC mRNAs (Kontula *et al.*, 1984; Kahana & Nathans, 1984; McConlogue *et al.*, 1984; Alhonen-Hongisto *et al.*, 1985a), the human cells only expressed, as it appears, a single mRNA species. This was actually confirmed by side-by-side blot analyses with Sultan and mouse L1210 RNAs.

Human myeloma cells develop ODC-gene amplification under the pressure of DFMO, as evidenced by increased enzyme activity, enhanced synthesis of the enzyme protein, dose-dependent increase in the expression of ODC mRNA and increased amount of hybridizable DNA sequences in drug-resistant cells. When compared with mouse cells (Ehrlich ascites, carcinoma, L1210 leukaemia) (Alhonen-Hongisto *et al.*, 1985a,c), the human cells of the present study undergo ODC-gene amplification at clearly lower DFMO concentrations, which may be partly due to their higher sensitivity to the anti-proliferative effects of DFMO.

The importance of gene amplification to the emergence of resistance to cancer chemotherapy agents and to the malignant process as well is becoming increasingly acknowledged. Besides necessitating carefully planned combined chemotherapy to circumvent possible developing drug resistance, it has been suggested that the gene-amplification-induced arrangements may play a role in the progression of the malignant process (Schimke, 1984). It has been speculated that agents which generate replication of DNA sequences may facilitate over-replication and the consequent generation of cells with the phenotype of loss of growth control (Mariani & Schimke, 1984). In line with this prediction, we have previously shown that Ehrlich ascites-carcinoma cells with ODC gene amplification become more aggressive and probably also more tumorigenic (Alhonen-Hongisto *et al.*, 1985b). We believe that further studies with human cell lines are highly desirable, as they could reveal changes that may be important, not only to the

metabolism of polyamines, but also to the very nature of human malignancies in general.

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