Long-term reduction of amplified ornithine decarboxylase sequences in human myeloma cells

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(1) Human myeloma cell line Sultan, resistant to 20 mM difluoromethylornithine (DFMO) owing to ornithine decarboxylase (ODC) gene amplification, was grown in the absence of DFMO for a period of 10 months. The gene copy number and methylation status of the ODC gene were monitored after withdrawal of DFMO. Moreover, levels of ODC mRNA, immunoreactive ODC protein, ODC activity and polyamine levels were recorded recurrently during the course of the study. (2) The results revealed that ODC gene copy number started to decrease after 4 weeks growth without DFMO, to a final level of less than 30 % of the original gene dosage. The methylation status of the ODC gene, however, remained almost unaltered, displaying only a modest increase in methylation after 10 months without DFMO. The amount of ODC message dropped very rapidly to 75 % of the original value, then started to decrease in a gene copy-number-dependent manner. The amount of ODC protein closely followed the levels of mRNA during the study, whereas the ODC activity, after a transient increase during the first week, decreased to half of the original level after 4 weeks. Between 6 and 16 weeks ODC activity stabilized to a fifth of the original value and no more changes were detected during the subsequent period of observation. (3) Due to the grossly elevated ODC enzyme activity, levels of putrescine and spermidine first peaked and then

stabilized at 6 weeks after DFMO withdrawal. The final spermidine level was comparable with that of the parental Sultan cell line with only one copy of active ODC gene. However, putrescine content was strikingly elevated, being stabilized to a level that was 20 times higher than in parental cells. Spermine concentration was practically unchanged during the study. (4) According to the results obtained in this study, the abnormal level of ODC expression in human myeloma cells is suppressed partially at the level of transcription or post-transcriptionally, but it is not due to increased methylation of the gene. The major regulatory mechanism to compensate for a highly elevated ODC expression was modulation of the enzyme activity. After 10 months without DFMO, the cells still displayed about 20 times higher ODC activity and putrescine concentration than the myeloma cell line with a single copy of the ODC gene. They did not, however, show any signs of growth retardation or other features different from the parental cells. Apparently the myeloma cell line used in this study can tolerate relatively high levels of putrescine, as this diamine apparently is not converted into higher polyamines. This finding is in accordance with studies dealing with transgenic mice over-expressing the human ODC gene.

INTRODUCTION

Cultured cancer cells can easily be adapted to grow in the presence of 2-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC). Cells can circumvent the inhibition of putrescine synthesis by a number of different mechanisms. One of the best-characterized mechanisms is the amplification of genomic ODC sequences resulting in a massive accumulation of ODC message as well as immunoreactive ODC protein. This ample excess of ODC enzyme can produce, even in the presence of 20 mM DFMO, a small amount of putrescine that is readily converted into higher polyamines, especially to spermine, thus enabling the cells to survive and even to proliferate at a normal rate.

Our previous studies with a human myeloma cell line revealed that acquired DFMO resistance developed owing to ODC gene amplification [1], which was accompanied by a hypomethylation at the ODC gene locus [2]. This cell line was further characterized in respect to polyamine biosynthetic and biodegradative enzymes, as well as to cellular polyamine pools [3] including a quantification of ODC gene copy number and mRNA levels [4]. Moreover, studies with clones derived from the parental myeloma cell

line and from the cell line with amplified ODC sequences were carried out to establish further the role of ODC gene hypomethylation in its amplification. The results indicated that a typical methylation pattern of the amplified ODC gene involved a hypomethylation of the 3' flanking region. Clones with this amplification-prone methylation pattern were also found among the parental cells [5].

Transfection experiments with the human ODC gene revealed that ODC expression can be suppressed by *in vitro* methylation [6]. This is obviously due to an increased number of methylated CpG sequences at the promoter region of the ODC gene that is normally completely unmethylated in every cell type studied so far [7]. On the other hand, the coding region of the gene is totally methylated, whereas the 3' flanking region displays variable extents of hypomethylation, especially in the amplified ODC gene. Whether the observed hypomethylation in the 3' flanking region of the human ODC gene is a cause or a consequence of the gene amplification process, is still unknown.

In the present study, cells resistant to 20 mM DFMO (owing to ODC gene amplification) were grown for 10 months in the absence of DFMO. Here we have monitored the gradual disappearance of amplified ODC sequences, the methylation status

of the gene, the rate of transcription of the genes as well as the accompanying changes of the intracellular polyamine pools upon withdrawal of DFMO.

EXPERIMENTAL

Cell cultures

The human myeloma cell line Sultan, which is resistant to 20 mM DFMO, has been characterized in detail in previous studies [1,3–5]. Cells were cultured in RPMI 1640 (Gibco, Paisley, Scotland, U.K.) supplemented with 5% fetal-calf serum (Gibco) and gentamicin (Sigma). After withdrawal of DFMO, the cells were harvested for monthly analyses, 24 h after dilution of the culture with fresh medium. The osmolality of 20 mM DFMO was measured with a Halbmikro osmometer (Knauer, Berlin, Germany). A solution of 30 mM NaCl appeared to contain the same osmotic value as 20 mM DFMO. A parallel culture with 30 mM NaCl instead of DFMO was maintained for the first 6 weeks and harvested identically.

Chemicals

DFMO was generously given by Dr. Nickolaus Seiler (Marion Merrel Dow, Strasbourg, France). L-[1-¹⁴C]Ornithine (specific radioactivity 61 Ci/mol) was purchased from Amersham International (Amersham, Bucks., U.K.). The restriction endonucleases *HpaII*, *MspI* and *HindIII* were from Boehringer-Mannheim (Mannheim, Germany) or New England Biolabs (Cleveland, OH, U.S.A.). The non-radioactive DNA labelling kit was purchased from Boehringer-Mannheim.

Analytical methods

Total cellular RNA was isolated by the method of Chomczynski & Sacchi [8] and the RNA was dissolved in water with 1 unit/ μ l of RNasin RNase inhibitor (Promega, Madison, WI, USA.). RNA (5 μ g) was fractionated on a 1.4% (w/v) agarose/formaldehyde gel and transferred to positively charged nylon filters (Boehringer-Mannheim). Northern blot analysis was carried out by the method of Engler-Blum et al. [9] by using digoxigenin-labelled probe pODC 10/2H complementary to human ODC cDNA [10]. The scanning of Northern-blot X-ray films was performed with a Hewlett-Packard ScanJet Plus scanner, Macintosh DeskScan 2.0 (Hewlett-Packard) program and Image 1.54 analysis software (NIH Research Service Branch).

Genomic DNA was isolated by the method of Blin and Stafford [11] and digested according to the instructions of the suppliers of the restriction enzymes. The restriction fragments were electrophoresed in a 0.9% agarose gel and the following steps, including transfer of DNA fragments to a membrane and hybridization, were performed as described above. Southern-blot X-ray films were scanned as described above and the 5 kbp *HindIII* fragment was used for the analysis of the ODC gene copy number.

A 10 μ g sample of protein was electrophoresed in an SDS/12% polyacrylamide gel and electroblotted on to Immobilon-P membrane (Millipore, U.S.A.). The blot was blocked for 30 min in PM-TBS [2% (w/v) non-fat dried milk, 10 mM Tris/HCl, pH 7.4, 150 mM NaCl] and incubated for 2 h with rabbit anti-human ODC antibody (generous gift from Dr. Antti Pajunen) diluted 1:500 in PM-TBS. The membrane was washed three times with TBS and incubated for 1 h with alkaline phosphatase-coated anti-(rabbit IgG) (Zymed, U.S.A.) diluted 1:1000 in PM-TBS. After three washes with TBS, the blot was developed by Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining [12]. The blot was scanned as described above.

The ODC activity was assayed by the method of Jänne and Williams-Ashman [13].

The concentrations of the polyamines were determined with the aid of HPLC (Hewlett-Packard, Waldbronn, Germany) by the method of Hyvönen et al. [14].

RESULTS

Genomic DNA was digested with restriction enzymes *HpaII*, *MspI* and *HindIII*, and Southern-blot analysis was performed as described in the Experimental section. The 5 kb fragment from *HindIII* digestion was used for determination of the ODC gene copy number. Enzymes *HpaII* and *MspI* are isoschizomers, both recognizing the sequence CCGG. *HpaII*, however, is unable to cut when the internal C is methylated, while *MspI* is insensitive to DNA methylation. The sizes of fragments, as well as their relative intensities, produced by *HpaII* can thus be used to determine the overall changes of DNA methylation in and around the sequences complementary to the probe.

Figure 1 displays the changes that have taken place in the ODC sequences in Sultan 20D myeloma cells after withdrawal of DFMO. The gene copy number appeared to be unchanged after 4 weeks growth without DFMO. The closer analysis of the very first weeks of reversion (results not shown) revealed that the number of ODC genomic sequences started to reduce after 6 weeks and thereafter decreased gradually, being less than 30% of the original value after 10 months without DFMO (Figure 2). This was still more than 20 times higher than the number of ODC gene copies present in the parental Sultan cell line. The methylation status of the ODC gene remained unaltered at least for the first 4 months of the experiment, even beyond the point where the reduction of the gene copy number was apparent. Yet, after 10 months of growth without DFMO the pattern of bands from *HpaII* digestion was the same, although the intensities of the two largest fragments had changed. This indicated a partial remethylation of at least one CCGG site in or around the ODC gene.

Levels of ODC mRNA were measured by scanning the intensities of the bands on X-ray films after the Northern blotting. As RNA easily undergoes degradation during prolonged storage (even at -70°C), the amount and condition of RNA

Figure 1 Restriction enzyme analysis of ODC sequences in DFMO-resistant myeloma cells grown without DFMO

DNA (10 μ g) from the parental Sultan cell line (Parental), from the DFMO-resistant cell line grown with 20 mM DFMO (20D) and from the DFMO-resistant cell line grown without DFMO for 24 h (24 h), for 1 month (30 d), for 4 months (120 d) and for 10 months (300 d), was digested with *HindIII* (Hi), *HpaII* (Hp) and *MspI* (Ms). Molecular size markers are shown on the left.

Figure 2 Reduction of genomic ODC sequences after the removal of DFMO from the growth medium

The 5 kbp *HindIII* fragment (Figure 1) was scanned as described in the Experimental section. The amount of ODC gene present in the Sultan 20D (S20D) cell line grown in 20 mM DFMO represents the 100% value. The basal amount of ODC gene in the parental Sultan cell line is also indicated (◆).

loaded on to the gel was checked by staining the identical gel with ethidium bromide and determining the intensities of the ribosomal RNA bands. Figure 3 shows the relative amount of ODC message after DFMO withdrawal. It appears that cells can reduce their mRNA levels very rapidly, but this contributed only about 25% of the final regulation of ODC activity. After this transient change of either ODC gene transcription or mRNA degradation, the message levels dropped in a gene-copy-number-dependent manner, representing about 20% of the amount present in cells grown in 20 mM DFMO. When compared with the parental Sultan cell line, this level was, again, still about 20 times higher.

Results from Western blots with ODC-specific antibody (Figure 4) indicated that the intracellular levels of immunoreactive ODC protein were tightly coupled to the levels of ODC message. Subsequently, the final protein level after 10 months

Figure 3 Reduction of ODC mRNA levels after the removal of DFMO from the growth medium

The 2 kbp message from Northern blot, the human ODC mRNA, was scanned as described in the Experimental section. The amount of ODC message present in the Sultan 20D (S20D) cell line grown in 20 mM DFMO represents the value 100% (◇). The basal amount of ODC message in the parental Sultan cell line is also indicated (◆).

Figure 4 Reduction of immunoreactive ODC protein after the removal of DFMO from the growth medium

The 50 kD band from Western blot representing the subunit of functional ODC protein was scanned as described in the Experimental section. The amount of ODC protein present in the Sultan 20D (S20D) cell line grown in 20 mM DFMO represents the value 100% (◇). The basal amount of ODC protein in the parental Sultan cell line is also indicated (◆).

growth without DFMO was about 20% of the original level and approximately 20 times higher than in the parental cell line.

ODC activity displayed changes that could not be seen in the levels of ODC gene, mRNA or protein (Figure 5). Namely, the enzyme activity was about 10% higher after 1 week without DFMO. This transiently elevated activity rapidly decreased during the next week to the same level as ODC protein. Over the next 3 weeks the ODC activity decreased to 50% of the original level and finally, after 4 months, the enzyme activity was stabilized at the 20% level. The results clearly indicate that the ODC enzyme activity was regulated by a mechanism that was independent of the gene copy number or the amount of message.

In contrast to results reported earlier [3] the present Sultan 20D cell line with ODC gene amplification did not contain measurable amounts of putrescine or spermidine 24 h after dilution with fresh medium. However, the spermine level was

Figure 5 Changes of ODC activity after the removal of DFMO from the growth medium

Specific ODC activity (pmol of CO_2 /mg of protein per h) was determined as described in the Experimental section. The amount of ODC activity present in the Sultan 20D (S20D) cell line grown in 20 mM DFMO represents the value 100% (◇). The basal amount of ODC activity in the parental Sultan cell line is also indicated (◆).

Figure 6 Changes of intracellular polyamine pools after the removal of DFMO from the growth medium

Polyamines were analysed with the aid of a HPLC method as described in the Experimental section: Δ , putrescine; $\blacklozenge$, spermidine; $\square$, spermine contents in Sultan 20D cell line grown in the absence of DFMO. As the spermidine and spermine contents in the parental Sultan cell line appeared to be identical to that at 10 months after DFMO withdrawal, only the basal level of putrescine is indicated ($\diamond$).

comparable with the parental Sultan cell line (Figure 6). After the withdrawal of DFMO, the intracellular putrescine level climbed extremely high and then, during the next 5 weeks, dropped to a final level which was about 20 times higher than that in the parental cell line. The spermidine level was also elevated rapidly, but not to the same extent as putrescine, whereas the spermine level stayed practically unchanged during the experiment. The final levels of both spermidine and spermine were comparable with the levels in the parental Sultan cell line. Despite of the highly elevated putrescine level, the cells growing without DFMO did not show any signs of altered morphology or growth rate.

DISCUSSION

In the present study we have followed the long-term changes accompanied by the reduction of stably amplified ODC gene sequences in a human myeloma cell line with acquired resistance to DFMO [3] upon withdrawal of the drug. Before the omission of DFMO from the culture medium, the cells contained about 100 ODC gene copies (the parental cell line only has a single gene copy), 100 times higher amounts of ODC mRNA, hnRNA and ODC activity [4]. These changes resulted in a highly unusual cellular polyamine pattern, as the myeloma cells growing in the presence of the inhibitor did not, in practice, contain any putrescine or spermidine, yet the cells were able to proliferate in a normal fashion in the presence of elevated concentrations of spermine as the only polyamine. Upon withdrawal of DFMO, the ODC gene dosage remained virtually unaltered for the first 4 weeks, whereafter a gradual reduction took place. However, even at the end of the 10-month observation period, the cells still contained about 30 copies of the ODC gene. The amount of ODC mRNA, and that of immunoreactive protein, dropped immediately to a level corresponding to about 75% of that present in the cells before the withdrawal of the drug, and then decreased in a gene-copy-number-dependent fashion (Figures 1–3). However, the most rapid changes occurred in the ODC activity, which was rapidly reduced to a final level corresponding to about 20% of the original activity, yet still representing 20 times higher activity in comparison with that found in the

parental cells (Figure 4). The initial stimulation of the enzyme activity immediately after the drug withdrawal was apparently attributable to a hypo-osmotic effect following the omission of 20 mM DFMO from the culture medium (Figure 5). The changes in the cellular polyamine pools were strikingly rapid. Immediately upon the withdrawal of DFMO, the concentration of putrescine increased from non-detectable to a very high level and was then stabilized to a level that was 20 times higher than that found in the parental myeloma cells (Figure 6). The rapid increase in putrescine upon withdrawal of DFMO apparently indicates that a large portion of ODC was enzymically active and only competitively inhibited by the drug. Similarly, the non-existing pool of spermidine was immediately restored to a level found in the parental cells after the omission of the drug, with only a small overshoot (Figure 6). The elevated pool of spermine was likewise normalized within a few weeks of withdrawal of the drug (Figure 6). It is remarkable that the cells then continued to grow, apparently normally, in the presence of a 20 times higher putrescine concentration compared with parental myeloma cells.

According to the present results, it appears that the abnormal ODC expression caused by gene amplification is initially suppressed upon withdrawal of the inhibitor through a mechanism involving a transcriptional component. The experiments carried out with the isoschizomeric restriction enzymes *HpaII* and *MspI* did not support the view that changes in ODC gene methylation would play a critical role in the initial reduction of the transcription rate. The fact that the decrease in the amount of immunoreactive ODC closely followed that of the accumulation of ODC message apparently rules out any significant contribution of translational control mechanisms to the rapidly decreased enzyme activity. It thus appears that the abnormally high ODC activity is suppressed upon the omission of the inhibitor by post-translational mechanisms that do not involve changes in the half-life of the enzyme (J. Wahlfors, S. Myöhänen, V.-P. Korhonen, L. Alhonen and J. Jänne, unpublished work). However, the remarkable fact remains that the biosynthesis of the polyamines is not nearly 'normalized' to the rate found in the parental cells, as the activity of ODC and the concentration of putrescine were stabilized to a level corresponding to about a 20-fold excess in comparison with the wild-type cells. In spite of the continuous massive putrescine overaccumulation the concentrations of spermidine and spermine remained at the level found in the parental cells and the revertant cells continued to grow with no apparent changes in their morphological appearance or proliferation rate. It thus appears that under these conditions the overaccumulated putrescine was not converted to the higher polyamines to any appreciable extent. Interestingly, the observed changes in the polyamine metabolism in the revertant myeloma cells closely resemble those found in the tissues of transgenic mice overexpressing the human ODC gene [15]. In these animals, strikingly elevated (up to 100-fold) ODC activity and massive tissue putrescine overaccumulation did not result in any changes in the pools of the higher polyamines. The apparent block of the conversion of putrescine into spermidine and spermine was not explicable by changes in the activities of the enzymes involved in polyamine metabolism such as *S*-adenosylmethionine decarboxylase or the backconversion enzymes spermidine/spermine acetyltransferase or polyamine oxidase [16]. It is likewise interesting that the revertant cells described in the present study apparently only reduced the activity of ODC and the accumulation of putrescine to a level sufficiently low to maintain the pools of spermidine and spermine within the normal range. Just as in the case of the transgenic animals overexpressing the ODC gene [15], living cells apparently tolerate very high concentrations of putrescine with no adverse effects on their proliferative capacity. A possible

exception to this rule seems to be male germ-line cells, as spermatogenesis is severely disturbed in transgenic mice with overt testicular ODC overexpression [15,17]. Taking together the present results and those obtained with the transgenic mice overexpressing ODC, as well as the recent report by Pitkin et al. [18] on *Neurospora* strains with excessive putrescine accumulation, there is strong support for the view that one of the major reasons for the tight regulation of polyamine metabolism in living cells is to prevent an aberrant accumulation of the higher polyamines spermidine and spermine, as also recently pointed out by Davis et al. [19]. One of the key features would thus be the elucidation of the nature of the block between putrescine and spermidine and eventually to release it in cultured cells or living animals overexpressing ODC.

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REFERENCES

- 1 Leinonen, P., Alhonen-Hongisto, L., Laine, R., Jänne, O. A. and Jänne, J. (1987) *Biochem. J.* **242**, 199–203
- 2 Alhonen-Hongisto, L., Leinonen, P., Sinervirta, R., Laine, R., Winqvist, R., Alitalo, K., Jänne, O. A. and Jänne, J. (1987) *Biochem. J.* **242**, 205–210
- 3 Hirvonen, A., Eloranta, T., Hyvönen, T., Alhonen, L. and Jänne, J. (1989) *Biochem. J.* **258**, 709–713
- 4 Hyttinen, J.-M., Halmekytö, M., Alhonen, L. and Jänne, J. (1991) *Biochem. J.* **278**, 871–874
- 5 Wahlfors, J. (1991) *Biochem. J.* **279**, 435–440
- 6 Halmekytö, M., Hirvonen, A., Wahlfors, J., Alhonen, L. and Jänne, J. (1989) *Biochem. Biophys. Res. Commun.* **162**, 528–534
- 7 Myöhänen, S., Wahlfors, J. and Jänne, J. (1994) *DNA Seq.-J. Seq. Map* **5**, 1–8
- 8 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 9 Engler-Blum, G., Meier, M., Frank, J. and Müller, G. A. (1993) *Anal. Biochem.* **210**, 235–244
- 10 Hickok, N. J., Seppänen, P., Gunsalus, G. and Jänne, O. A. (1987) *DNA* **6**, 179–187
- 11 Blin, N. and Stafford, D. W. (1976) *Nucleic Acids Res.* **3**, 2303–2308
- 12 Harlow, E. and Lane, D. (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory, New York
- 13 Jänne, J. and Williams-Ashman, H. G. (1971) *J. Biol. Chem.* **246**, 1725–1732
- 14 Hyvönen, T., Keinänen, T. A., Khomutov, A. R., Khomutov, R. M. and Eloranta, T. O. (1992) *J. Chromatogr.* **574**, 17–21
- 15 Halmekytö, M., Hyttinen, J.-M., Sinervirta, R. et al. (1991a) *J. Biol. Chem.* **266**, 19746–19751
- 16 Halmekytö, M., Alhonen, L., Wahlfors, J. et al. (1991b) *Biochem. J.* **278**, 895–898
- 17 Hakovirta, H., Keiski, A., Toppari, J., et al. (1993) *Mol. Endocrinol.* **7**, 1430–1436
- 18 Pitkin, J., Perriere, M., Kanhel, A., Ristow, J. L. and Davis, R. H. (1994) *Arch. Biochem. Biophys.* **315**, 153–160
- 19 Davis, R. H., Morris, D. R. and Coffino, P. (1992) *Microbiol. Rev.* **56**, 280–290

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