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Asparagine markedly induces the expression of ornithine decarboxylase gene in transformed mammalian cells but not in their untransformed counterparts

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Abstract

We have previously shown that asparagine alone induces a 10–15-fold increase in ornithine decarboxylase (ODC) mRNA level in DF-40 mouse neuroblastoma cells. The induction is due to an accumulation of ODC mRNA through a post-transcriptional stabilization mechanism (Chen, Z.P. and Chen, K.Y. (1992) J. Biol. Chem., 267, 6946–6951). In the present study we showed that asparagine induced ODC gene expression in v-Ha-*ras*-transformed 3T3 (*ras*-3T3) cells but not in 3T3 cells. Other growth related genes including c-*src*, c-*ras*, and c-*fos* were not affected by asparagine in *ras*-3T3 cells. Southern blot analysis indicated that the pronounced asparagine effect was not due to ODC gene amplification in *ras*-3T3 cells. The effect of asparagine on the induction of ODC mRNA could account for the significant increases in the ODC activity in *ras*-3T3 cells. We also examined the effect of asparagine on ODC gene expression in human KD cells and their transformed counterparts. Our findings strongly suggest that the induction of ODC mRNA by asparagine may represent a component of an altered growth regulatory program associated most prominently with cell transformation.

Keywords: Asparagine; Ornithine decarboxylase; Transformed cells; Gene expression

1. Introduction

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the key enzyme for the biosynthesis of polyamines [1–3]. The importance of polyamines in the growth and survival of living organisms has been well documented [1–4]. Mammalian ODC activity increases dramatically in response to a whole array of hormonal, developmental, and growth related stimulation [5,6]. Besides serum and other growth-stimulatory agents, asparagine alone can induce maximal ODC activity in N18 mouse neuroblastoma cells and other cell lines [7–12]. ODC gene is serum-responsive and cell cycle-dependent [1–6], and can be regulated at transcriptional, translational, and post-translational levels [13].

We have recently investigated the mechanism of the asparagine-mediated ODC induction in a

Abbreviations: EBSS, Earle's balanced salt solution; ODC, ornithine decarboxylase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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mouse neuroblastoma DF-40 cell line. We demonstrated that asparagine alone could induce a 15-fold increase in the level of ODC mRNA, primarily due to post-transcriptional stabilization of ODC mRNA [14]. In addition, asparagine specifically stimulates the translation of ODC mRNA and increases the half-life of ODC protein [14]. These three effects account for the 200-fold increase in ODC activity in DF-40 cells. In contrast, Kanamoto et al. [11] reported that asparagine has little effect on the level of ODC mRNA in rat hepatocytes. We speculated that the apparent discrepancy could be due to a difference in cell type used in these two studies. Since DF-40 mouse neuroblastoma is a tumor cell line whereas the rat hepatocytes are a normal primary cell strain, it is possible that these two different cell types may respond to asparagine differently. It is well known that chemically and virally transformed cells in tissue culture show increased ODC activity and polyamine accumulation when compared with their normal counterparts [15–17]. Therefore, the effect of asparagine in inducing ODC mRNA may be more pronounced in transformed cells than in their normal counterparts. To test this possibility, we have compared the effect of asparagine on the induction ODC gene expression in transformed cell lines and in their normal or untransformed counterparts. Our results suggest that the induction of ODC mRNA by asparagine may be a phenotype associated with cell transformation.

2. Material and methods

2.1. Materials

All tissue culture supplies were obtained from Gibco (Grand Island, NY). L-Asparagine and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Molecular biological grade of SDS, agarose and [α-^{32}P]dCTP (3000 Ci/mmol) were from ICN Chemical, Radioisotope Division, Irvine, CA. L-[1-^{14}C]Ornithine (54.3 mCi/mmol) was from DuPont New England Nuclear (MA). *Eco*RI, *Hin*dIII, *Bam*HI, RNAase, proteinase K and nick translational kits were from BRL (Bethesda, MD).

2.2. Cell culture and enzyme induction

Mouse NIH3T3 cells and EJ *ras* bladder carcinoma oncogene transformed NIH3T3 cells were given by Dr E.S. Canellakis, Yale Medical School (New Haven, CT). KD and HuT13, HuT14 human fibroblastic cells were given by Dr John Leavitt, California Institute of Medical Research (Palo Alto, CA) [18]. All transformed cell lines showed characteristic substratum-independent growth in soft agar. Mouse cells were maintained in Dulbecco's medium supplemented with 15% new-born calf serum at 37°C in a water-jacketed Forma CO_2 incubator. Human cells were grown in the presence of 10% fetal bovine serum. Subcultivation at 1:5 dilution was performed after cell cultures reached confluence. For the enzyme induction, cultures at 90% confluence were serum-deprived for 3 h and then incubated in EBSS containing 10 mM L-asparagine at 37°C for various lengths of time as indicated. At the end of incubation, cells were washed three times with cold phosphate buffered saline.

2.3. Enzyme assay and RNA analysis

For ODC activity assay, cells were pelleted and resuspended in TEPD buffer (50 mM TRIS–HCl, pH 7.4, 0.1 mM EDTA, 50 mM pyridoxal phosphate and 5 mM dithiothreitol) and homogenized by sonication. ODC activity was determined by procedure previously described [7]. Total cellular RNA was prepared from $1-3 \times 10^7$ cells according to the procedure previously described [14]. Poly(A)$^+$ RNA was isolated from total RNA using oligo (dT)-cellulose column. Either total cellular RNA or poly(A)$^+$ RNA was analysed by Northern blot analysis as previously described [14]. The cDNA probes used were pODC 54 from Dr O. Janne [19] and pMc-*myc* 54 from Dr Marcu [20]. Other oncogene probes were obtained from American Type Culture Collection (Rockville, MD).

2.4. Isolation of genomic DNA and Southern blot analysis

High molecular weight genomic DNA was isolated by a procedure described by Arrand [21]. Restriction endonuclease digestion was carried out

following the conditions described by manufacturers. The restricted DNA fragments were separated by electrophoresis on a 0.8% agarose gel under denaturing conditions. DNA gel was then transblotted on a Gene Screen Plus membrane. The membrane was hybridized with ^{32}P-labeled pODC54.

3. Results

3.1. Induction of ODC activity in 3T3 and v-Ha-vas-transformed 3T3 cells

Fig. 1A shows the time course of ODC activity induced by asparagine in NIH3T3 and *ras*-transformed NIH3T3 cells maintained in the salts/glucose solution. Fig. 1B shows the induction of ODC activity by serum in both cell types. The peak ODC activity induced by asparagine in *ras*-3T3 cells was about 40 units/mg protein, comparable with the maximal level of ODC activity (~46 units/mg protein) induced by serum in *ras*-3T3 cells. In contrast, the peak activity in 3T3 cells induced by asparagine and by serum was, respectively, 7 units/mg protein and 16 units/mg protein. The results indicate that a single amino acid, asparagine, is as effective as serum in inducing maximal ODC activity in 3T3 and *ras*-3T3 cells. Moreover, the data showed that, similar to serum, the effect of asparagine on ODC induction was more pronounced in transformed cells than that in normal cells. The kinetics of ODC induction by serum in 3T3 and in *ras*-3T3 cells was similar; in both cases ODC activity peaked 3 h after stimulation (Fig. 1B). However, ODC activities induced by asparagine in 3T3 cells and *ras*-3T3 cells exhibited somewhat different time course (Fig. 1A), suggesting different mechanism of induction.

3.2. Effect of asparagine on the expression of ODC and other oncogenes

Fig. 2 shows that asparagine alone induced a large increase in ODC mRNA in *ras*-3T3 cells but not in 3T3 cells. The size of ODC mRNA was approximately 2.2 kb in both 3T3 and *ras*-3T3 cells,

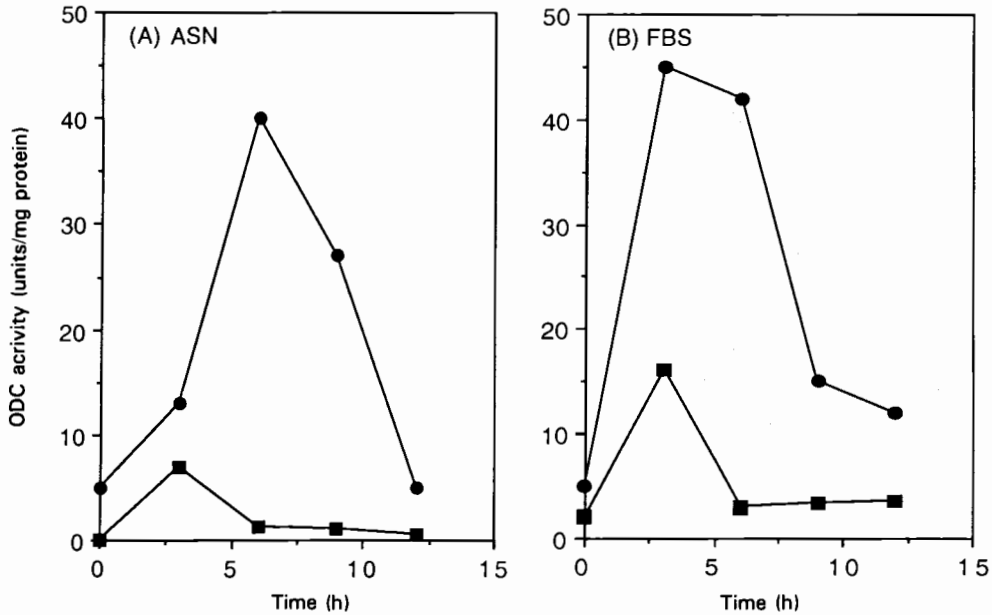

Fig. 1. Induction of ODC activity in 3T3 and *ras*-3T3 cells. Confluent 3T3 (■) and *ras*-3T3 (●) cells were serum-deprived for 3 h and then incubated either in fresh Dulbecco's medium plus 10% fetal bovine serum or in fresh EBSS plus 10 mM asparagine. The cultures were incubated for various times as indicated and then harvested for ODC activity assay. (A) ASN, asparagine treated; (B) FBS, fetal bovine serum treated cultures.

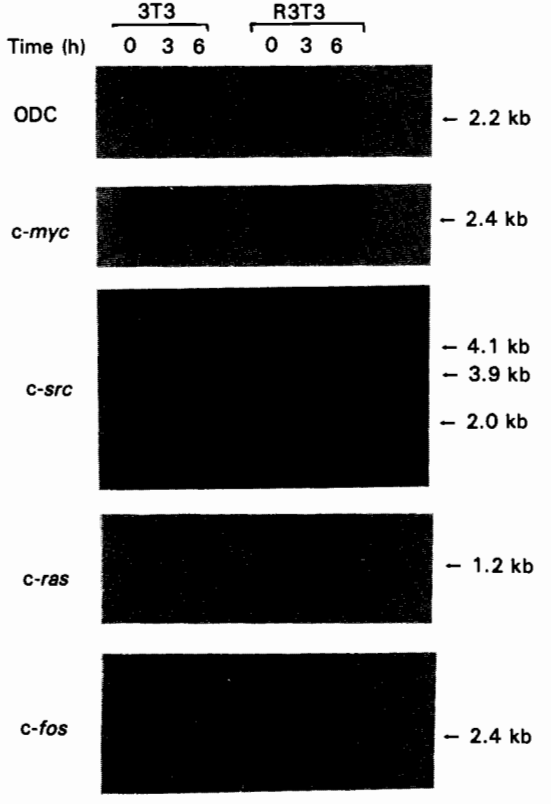

Fig. 2. Effect of asparagine on the expression of various growth-related genes in 3T3 cells and in *ras*-3T3 (R3T3) cells. Confluent cultures were serum-deprived for 3 h and then incubated in fresh EBSS containing 10 mM asparagine. Cells were harvested at various times as indicated and total RNA isolated for Northern blot analysis as described in *Materials and methods*. DNA probes used for hybridization were: pODC54 for ODC, pMc-myc54 for c-*myc*, pfos-1 for c-*fos*, the c-Ha-*ras* *Bam*H1 insert, and the pBR322 containing 2.95 kb src for c-*src*. Each lane contained 20 μg of total RNA.

similar to that reported in other mouse cell lines [19]. Quantitative estimation of the steady state levels of ODC mRNA by densitometric tracing of the Northern blot showed that asparagine caused a 6-fold increase in ODC mRNA level in *ras*-3T3 cells but had almost no effect on 3T3 cells. After 6 h of asparagine treatment, the ODC mRNA level in *ras*-3T3 cells was about 20-fold higher than that in 3T3 cells. Thus, the difference in ODC mRNA alone between 3T3 and *ras*-3T3 cells was sufficient to account for the difference in enzyme activities, indicating that asparagine-mediated ODC gene expression is different between normal and transformed cells at transcriptional or post-transcriptional level. The unique effect of asparagine on ODC gene expression prompted us to examine whether asparagine also affect expressions of other growth related genes. The results shown in Fig. 2 also indicated that, except perhaps for c-*myc*, asparagine had little or no effect on the expression of c-*fos*, c-*ras*, and c-*src*. Asparagine did not induce c-*erb*B, β-actin and thymidine kinase gene either (data not shown). Asparagine did induce c-*myc* gene expression 2-fold in *ras*-3T3 cells over a 6-h period. We have recently observed a 5-fold increase of c-*myc* mRNA level induced by asparagine in mouse neuroblastoma cells (Z.P. Chen and K.Y. Chen, unpublished data). The mechanisms underlying this induction and the significance of this observation are under investigation.

3.3. Southern blot analysis of ODC gene in 3T3 and ras-3T3 cells

Since co-amplification of ODC gene with other genes has been demonstrated in some drug-resistant cells [22], it is possible that there was an amplification of ODC gene in v-Ha-*ras*-transformed 3T3 cells, and such amplification could account for the enhanced ODC gene expression in *ras*-3T3 cells. To assess this possibility, we have carried out Southern blot analysis of ODC gene in both 3T3 and *ras*-3T3 cells as shown in Fig. 3. The restriction maps obtained with *Bam*HI and *Eco*RI for both 3T3 and *ras*-3T3 cells were identical to that reported for mouse cell lines [23,24]. The intensities of hybridized DNA bands were about equal for 3T3 and *ras*-3T3 cells, indicating that no ODC gene amplification occurred in *ras*-3T3 cells.

3.4. Effect of asparagine on ODC gene expression in other cell types

To examine whether the differential effect of asparagine in normal and in transformed cells may be a general phenomenon; we also examined the effect of asparagine on ODC gene expression in two transformed human fibroblastic cell lines, HuT13 and HuT14, and in a normal human diploid fibroblastic KD cells. Results summarized in Fig. 4 show that asparagine markedly induced

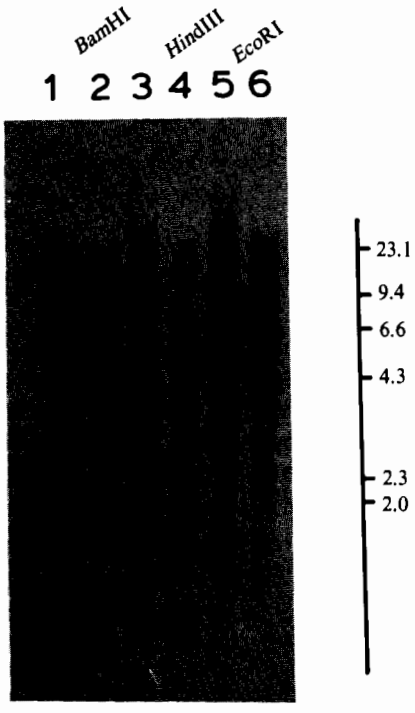

Fig. 3. Southern blot analysis of DNA from 3T3 and *ras*-3T3 cells. Genomic DNA from 3T3 (lanes 1, 3, and 5) and *ras*-transformed 3T3 (lanes 2, 4, and 6) were isolated, digested with *Eco*RI, *Hin*dIII, or *Bam*HI endonuclease. They were then fractionated on 0.8% agarose gel by electrophoresis. Hybridization was carried out using pODC54 probe. The size was determined by using *Hin*dIII digested λDNA as standard marker.

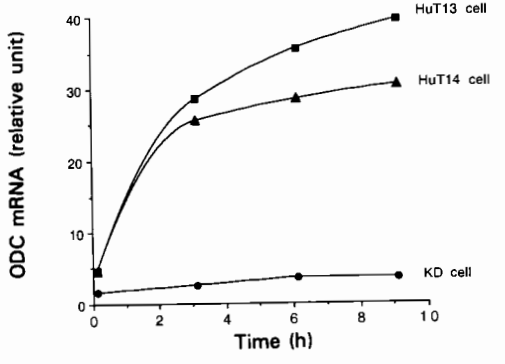

Fig. 4. Effect of asparagine on the induction of ODC mRNA in transformed human cell lines, HuT13 and HuT14, and in normal diploid KD cells. Confluent cells were serum deprived for 48 h and then incubated in fresh EBSS containing 10 mM of asparagine. Cells were harvested at time indicated for Northern blot analysis.

ODC gene expression at mRNA level in both transformed cells but had little effect in their normal counterparts. After 6 h of asparagine treatment, ODC mRNA level in HuT13 and HuT14 cells was, respectively, 26-fold and 24-fold higher than that in normal KD cells.

4. Discussion

ODC is the key enzyme for the biosynthesis of polyamines in mammalian cells [1–4]. The induction of ODC enzyme generally correlates with cell proliferation, and the inhibition of polyamine biosynthesis usually leads to growth inhibition [1–4]. Transformed cells in tissue culture generally show increased ODC activity and polyamine accumulation when compared with their normal counterparts [15–17].

We have previously shown that asparagine is necessary and sufficient to induce maximal ODC activity in various tumor cell lines maintained in a simple salt/glucose solution such as EBSS [7–9]. We and others have also shown that in primary cultures such as hepatocytes [11] or differentiated mouse neuroblastoma cells maintained in the salts/glucose solution, the maximal induction of ODC activity requires the presence of both asparagine and serum, or growth factors [10–12]. In all cases examined, serum or growth factors alone cannot induce ODC activity if cells are maintained in salts/glucose solution [7–12]. The effect of asparagine is specific; other A and N system amino acids are much less effective [7,8,14]. The present study confirms these earlier findings by demonstrating that asparagine alone can induce maximal ODC activity in transformed cells to the extent comparable with that induced by serum in complete medium (Fig. 1A,B). Our study now demonstrates that the induction of ODC activity by asparagine in *ras*-transformed 3T3 cells is more pronounced than in 3T3 cells (Fig. 1B), providing further evidence that asparagine is more effective in inducing ODC activity in transformed cells than in their normal counterparts. Since asparagine induces maximal ODC activity in cells maintained in simple salts/glucose solution instead of complex growth medium [7,14], the induction of ODC by asparagine offers a simple system for probing the

molecular basis of the difference between normal and transformed cells in ODC regulation.

We have shown that in DF-40 mouse neuroblastoma cells, asparagine induces ODC gene expression by (i) an induction of ODC mRNA (10–15-fold), (ii) a 4-fold increase in translation, and (iii) a 2–4-fold increase in the half-life of ODC protein [14]. Kanamoto et al. [11] reported that asparagine induced ODC mRNA in rat hepatocytes only 1.3-fold. The present study with normal and transformed cells (Figs. 2 and 4) clearly demonstrates that asparagine induces ODC mRNA in transformed cells but not in their untransformed or normal counterparts. The effect of asparagine on ODC mRNA appears to be the major cause for the difference in ODC activities between transformed *ras*-3T3 cells and 3T3 cells (Figs. 1 and 2). Thus, while translation and post-translational stimulation may account for the effect of asparagine on ODC activity in normal cells, the induction of ODC mRNA by asparagine appears to be a phenotype associated only with transformed cells.

The action of asparagine is quite specific; among other growth related genes examined, only c-*myc* was slightly induced by asparagine in *ras*-3T3 cells (Fig. 2). The effect of asparagine on c-*myc* induction is more pronounced in DF-40 mouse neuroblastoma cells (Z.P. Chen and K.Y. Chen, unpublished data). It is of interest to note that similar to ODC gene, c-*myc* gene also shares an AT rich core sequence ATTTA, a putative destabilizing sequence, in its 3′-untranslated region [25].

It is recognized that dietary constituents may specifically modulate gene expression by affecting gene transcription, mRNA processing, mRNA stability, and mRNA translation [26]. Although the precise physiological role of asparagine on ODC gene expression remains to be investigated, the marked difference between normal and transformed cells in response to asparagine allows us to use this system as a model to probe differences in biochemistry between normal and transformed cells. It may be also of interest to note cancer cells generally have a reduced requirement for serum during growth in tissue culture [27]. Considering the importance of ODC and polyamines to cell proliferation [4–6], the reduced serum-dependency of tumor cells could be explained at least in part by the fact that nutrients such as asparagine may replace some of the actions of serum such as the induction of ODC mRNA.

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