Characterization of a transgenic mouse line over-expressing the human ornithine decarboxylase gene

Maria HALMEKYTÖ, Leena ALHONEN, Jarmo WAHLFORS, Riitta SINERVIRTA, Terho ELORANTA and Juhani JÄNNE*

Department of Biochemistry and Biotechnology, University of Kuopio, P.O.B. 1627, SF-70211 Kuopio, Finland

We have produced several transgenic mouse lines over-expressing the human ornithine decarboxylase (ODC) gene. We have now characterized one of the transgenic lines as regards the tissue accumulation of the polyamines and the activities of their metabolizing enzymes. Among the tissues analysed, the polyamine pattern was most strikingly changed in testis and brain of the transgenic animals. ODC activity was greatly enhanced in all tissues, except kidney, of the transgenic animals. The most dramatic increase, 80-fold, was found in brain of the transgenic mice. The activities of *S*-adenosylmethionine decarboxylase and spermidine and spermine syntheses were likewise significantly increased in testis of the transgenic animals. The activities of the enzymes involved in the back-conversion of the polyamines, namely spermidine/spermine acetyltransferase and polyamine oxidase, were similar in the transgenic and non-transgenic animals. As analysed by reverse transcriptase/polymerase chain reaction, all the six tissues of the transgenic animals expressed human-specific ODC mRNA. Determination of the half-life of testicular ODC revealed a stabilization of the enzyme in the transgenic males.

INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17), the key enzyme in the biosynthesis of the polyamines, is a growth-related enzyme with a complex regulation (Hayashi, 1989). The view that ODC, and hence the accumulation of the polyamines putrescine, spermidine and spermine, is intimately involved in the growth processes of mammalian cells is supported by two lines of experimental evidence. Firstly, specific inhibition of ODC resulting in a disappearance of cellular putrescine and spermidine, but not spermine, invariably leads to a growth arrest of mammalian cells (Heby & Jänne, 1981). Secondly, an overproduction of ODC owing to gene amplification appears to give certain growth advantages, such as ability to grow in semi-solid media, at least for tumour cells (Alhonen-Hongisto *et al.*, 1985; Polvinen *et al.*, 1988).

The high level of conservation of ODC gene structure among mammalian species (Kontula *et al.*, 1984; Wen *et al.*, 1989; Fitzgerald & Flanagan, 1989; Hickok *et al.*, 1990) also suggests that this enzyme may play a critical role in the metabolism of animal tissues. We have been especially interested in human ODC. We isolated and sequenced the human chromosome-2-derived ODC gene (Hickok *et al.*, 1990) and showed in transfection experiments that this gene is expressed both stably (Hölttä *et al.*, 1989) and transiently (Halmekytö *et al.*, 1989) in Chinese-hamster ovary cells devoid of endogenous ODC activity. The transfected gene also appeared to retain its normal regulation, such as serum stimulation, short half-life and sensitivity to the polyamines (Hölttä *et al.*, 1989).

The isolation of the active human gene made it possible to produce transgenic mice carrying the human ODC gene. Even though the human and rodent ODC genes are highly similar, it is possible to detect the human gene in mice, owing to differences in their intron structures (Hickok *et al.*, 1990). So far we have produced several transgenic mouse lines over-expressing the human ODC gene. Most of these transgenic lines showed highly aberrant ODC expression, with very high activity in tissues such

as testis and brain. The high testicular ODC activity was associated with changes in testicular morphology, impaired spermatogenesis, and ultimately resulted in male infertility (Halmekytö *et al.*, 1991). We have now characterized one of the transgenic mouse lines (line K2 in Halmekytö *et al.*, 1991) aberrantly expressing the human ODC transgene. The most profound changes in polyamine levels and in the activities of their biosynthetic enzymes were found in testis and brain. The extremely high putrescine content in the testis of the transgenic animals may be causally related to the impaired spermatogenesis found in these animals. There were no signs whatsoever to indicate an activation of the back-conversion pathway of the polyamines as judged by the unaltered activities of spermidine/spermine acetyltransferase and polyamine oxidase in the transgenic animals. The transgene-derived ODC in testis had about twice as long a half-life as the corresponding mouse enzyme.

EXPERIMENTAL

Transgenic animals

The transgenic mice were produced by the standard pronuclear microinjection technique (Hogan *et al.*, 1986). Fertilized oocytes were obtained from superovulated BALB/c × DBA/2 mice mated with males of the same strain. The gene construct used for the microinjections was human ODC gene containing, in addition to the 12 exons and 11 intervening sequences, some 800 nucleotides of the 5' flank and 1000 nucleotides of the 3' flank of the gene. The primary detection of the transgene was accomplished with a PCR assay by using human-specific primers targeted to the third and fourth introns of the human ODC gene (the 5' primer 5'-GGCTTACATGTCTTGTTATGGAATGTAGAA-3' and the 3' primer 5'-GCTATCCATATGTGGCTTAACACGTGG-3'). The presence of the transgene was subsequently confirmed by detecting human-specific *Hind*III and *Msp*I restriction fragments in Southern-blot analysis. Animals of F2

Abbreviation used: ODC, ornithine decarboxylase.

* To whom correspondence should be addressed.

Table 1. Concentrations of putrescine, spermidine and spermine in tissues of transgenic and non-transgenic mice

Results are means $\pm$ S.D. from three animals in the group. tg–, non-transgenic; tg+, transgenic. Significance of the differences: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Tissue		Content (nmol/g wet wt.)			Spermidine Spermine
		Putrescine	Spermidine	Spermine	
Testis	tg–	16 $\pm$ 1.8	332 $\pm$ 26	591 $\pm$ 20	0.56 $\pm$ 0.02
	tg+	348 $\pm$ 45***	512 $\pm$ 26***	555 $\pm$ 12	0.92 $\pm$ 0.05***
Brain	tg–	< 5	302 $\pm$ 21	280 $\pm$ 6	1.08 $\pm$ 0.08
	tg+	62 $\pm$ 3***	385 $\pm$ 25*	286 $\pm$ 13	1.35 $\pm$ 0.08*
Spleen	tg–	46 $\pm$ 6	1020 $\pm$ 55	804 $\pm$ 39	1.26 $\pm$ 0.13
	tg+	86 $\pm$ 7**	1010 $\pm$ 8	767 $\pm$ 25	1.32 $\pm$ 0.06
Heart	tg–	< 5	130 $\pm$ 15	285 $\pm$ 30	0.46 $\pm$ 0.02
	tg+	17 $\pm$ 6***	126 $\pm$ 14	312 $\pm$ 36	0.41 $\pm$ 0.09
Kidney	tg–	20 $\pm$ 6	273 $\pm$ 22	625 $\pm$ 28	0.44 $\pm$ 0.05
	tg+	26 $\pm$ 4	339 $\pm$ 30*	603 $\pm$ 22	0.56 $\pm$ 0.05*
Liver	tg–	< 5	739 $\pm$ 70	852 $\pm$ 51	0.84 $\pm$ 0.12
	tg+	10 $\pm$ 4	831 $\pm$ 59	764 $\pm$ 43*	1.09 $\pm$ 0.03*

Table 2. Enzyme activities involved in the metabolism of the polyamines in tissues of transgenic and non-transgenic mice

Results are means $\pm$ S.D. from four animals in the group. tg–, non-transgenic; tg+, transgenic. Activities are expressed as pmol/h per mg wet wt., except for polyamine oxidase, as nmol/h per mg of protein. Significance of the differences: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Tissue		ODC	S-Adenosylmethionine decarboxylase	Spermidine synthase	Spermine synthase	Spermidine/spermine acetyltransferase	Polyamine oxidase
Testis	tg–	23 $\pm$ 4	22 $\pm$ 5	74 $\pm$ 17	44 $\pm$ 4	19 $\pm$ 1	2.32 $\pm$ 0.20
	tg+	629 $\pm$ 75***	53 $\pm$ 6***	125 $\pm$ 18**	72 $\pm$ 12**	20 $\pm$ 2	1.99 $\pm$ 0.27
Brain	tg–	0.4 $\pm$ 0.4	45 $\pm$ 3	141 $\pm$ 7	103 $\pm$ 15	14 $\pm$ 1	3.06 $\pm$ 0.33
	tg+	32 $\pm$ 4***	31 $\pm$ 2***	186 $\pm$ 13***	134 $\pm$ 21	13 $\pm$ 1	3.51 $\pm$ 0.31
Spleen	tg–	6 $\pm$ 2	30 $\pm$ 9	294 $\pm$ 33	86 $\pm$ 16	18 $\pm$ 3	4.04 $\pm$ 0.55
	tg+	41 $\pm$ 6***	23 $\pm$ 2	272 $\pm$ 13	75 $\pm$ 9	22 $\pm$ 7	3.84 $\pm$ 1.20
Heart	tg–	5 $\pm$ 1.7	7 $\pm$ 1.6	34 $\pm$ 4	52 $\pm$ 11	11 $\pm$ 5	0.60 $\pm$ 0.12
	tg+	40 $\pm$ 17***	3 $\pm$ 1.1**	32 $\pm$ 5	54 $\pm$ 9	9 $\pm$ 3	0.57 $\pm$ 0.1
Kidney	tg–	1440 $\pm$ 939	15 $\pm$ 3	83 $\pm$ 15	38 $\pm$ 4	6 $\pm$ 1	1.64 $\pm$ 0.25
	tg+	842 $\pm$ 491	11 $\pm$ 4	108 $\pm$ 3*	40 $\pm$ 4	7 $\pm$ 1	1.87 $\pm$ 0.17
Liver	tg–	0.3 $\pm$ 0.51	18 $\pm$ 3	295 $\pm$ 40	6 $\pm$ 1	6 $\pm$ 1	0.31 $\pm$ 0.04
	tg+	9 $\pm$ 6*	16 $\pm$ 1.3	285 $\pm$ 28	7 $\pm$ 2	7 $\pm$ 1	0.30 $\pm$ 0.04

Fig. 1. Detection of human specific ODC mRNA in tissues of transgenic mice

Total RNA was isolated from the tissues and used as the template for reverse transcriptase. The cDNA was amplified by using human-specific primers. The following dilutions were used for each tissue: undiluted (left), 1:10 (middle) and 1:100 (right). MW, molecular-size markers (pBR328 digested with *Bgl*I and *Hinf*I); bl, blank (no cDNA synthesis); C, control (total RNA from human Sultana myeloma cells).

generation derived from a transgenic founder animal designated as tg(ODC0HSA)K2 were used in all experiments. Their non-transgenic littermates served as controls.

Materials

L-[1-¹⁴C]Ornithine (sp. radioactivity 61 Ci/mol), DL-[1-¹⁴C]-methionine (sp. radioactivity 50 Ci/mol), [¹⁴C]spermine (sp. radioactivity 100 Ci/mol), [1-¹⁴C]acetyl-CoA (sp. radioactivity 54 Ci/mol) and 5'-[α -³²P]dCTP (sp. radioactivity 410 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.). Radioactive decarboxylated adenosylmethionine (substrate for propylamine transferases) was prepared by the method of Pösö *et al.* (1976) and further purified by Cellex-P chromatography (Eloranta *et al.*, 1976). The dNTPs used for PCRs were purchased from Pharmacia (Bromma, Sweden) and *Taq* polymerase was from Promega (Madison, WI, U.S.A.). Primer oligonucleotides were synthesized with a 381 A DNA synthesizer (Applied Biosystems, Warrington, Cheshire, U.K.), and PCRs were carried in a Hybaid thermal reactor (Hybaid, Teddington, Middx., U.K.).

Analytical methods

Polyamine concentrations were determined with a Hewlett-Packard HP 1090 liquid chromatograph by using the prechromatography procedure described by Mach *et al.* (1981). Total RNA was isolated by the method of Chomczynski & Sacchi (1987).

The activities of ODC and *S*-adenosylmethionine decarboxylase were assayed by the methods of Jänne & Williams-Ashman (1971a,b). The activities of spermidine and spermine synthases were assayed by the method of Raina *et al.* (1983), and that of polyamine oxidase was assayed by the method of Hölttä (1977). The activity of spermidine/spermine acetyltransferase was assayed by the method of Matsui *et al.* (1981).

The detection of human-specific ODC mRNA in the tissues of transgenic mice was accomplished with the combined reverse transcriptase/PCR assay as described by Hyttinen *et al.* (1991).

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

RESULTS

The concentrations of putrescine, spermidine and spermine in six different tissues of transgenic male mice and their non-transgenic littermates are depicted in Table 1. The most profound changes were found in testis and brain of mice carrying the human ODC transgene. Putrescine concentration in testis of the transgenic animals was more than 20 times that in their non-transgenic littermates. Similarly, brain putrescine level, which was below the detection limit in normal mice, was very high in the transgenic animals. Spermidine concentration was significantly elevated in testis, brain and kidney of the transgenic animals. Except for spleen and heart, the molar ratio of spermidine to spermine was significantly elevated in the tissues of transgenic mice.

Table 2 lists the activities of the enzymes directly involved in the metabolism of the polyamines in the same six tissues of the transgenic and non-transgenic animals. Except for kidney, ODC activity was grossly enhanced in all of the tissues of the transgenic animals, brain tissue displaying the largest increase, 80-fold. The activities of the rest of the enzymes showed only minor changes, except in testis, in which the whole biosynthetic pathway appeared to be activated (Table 2). The two enzymes, spermidine/spermine acetyltransferase and polyamine oxidase, engaged with the catabolism and back-conversion of spermidine and spermine,

Fig. 2. Half-life of testicular ODC in transgenic and non-transgenic mice

Each animal received cycloheximide (0.8 mg/100 g body wt.) intraperitoneally and was killed at the time points indicated. There were two animals per group. The ODC activity in the transgenic mice represents total testicular activity minus the endogenous mouse activity

did not show any changes in the tissues of the transgenic mice in comparison with their normal littermates.

Fig. 1 shows the levels of human-specific ODC in RNA in the same six tissues of transgenic mice as revealed by combined reverse transcriptase/PCR assay. Scanning of the picture revealed that testicular tissue contained the highest amount of human-specific ODC mRNA (about 10 times that in kidney and liver), thus roughly reflecting the differences in enzyme activities (Table 2).

We also determined the turnover rate of the transgene-derived ODC in the mice. Experiments with cultured human and mouse cells indicated that there was no difference between human and mouse enzyme as regards their molecular half-life, which ranged between 20 and 30 min (results not shown). Fig. 2 shows the decay of testicular ODC activity after cycloheximide treatment in normal and transgenic mice (note that the endogenous mouse activity is subtracted from the total ODC activity in the transgenic mice). It is obvious that the transgene-derived ODC was stabilized to a certain extent in comparison with the normal mouse enzyme. In fact, the half-life was just about doubled in the transgenic mice. The reasons for the apparent stabilization of the transgene-derived ODC are not known, but could be related to the possibility that human and mouse ODC structures are dissimilar enough to make the mouse ODC-degrading system less effective. This change, however, hardly explains the nearly 30 times higher ODC activity found in the testicular tissue of the transgenic males. A stabilization of the transgene-derived ODC as a general reason for the aberrant expression is rendered unlikely by the fact that there were great differences among the tissues as regards the extent of the enhancement of the enzyme activity (ranging from 0.6-fold in kidney to 80-fold in brain).

DISCUSSION

The reasons for the aberrant expression of the human ODC gene in transgenic animals are not obvious. Several potential causes can be excluded with a reasonable certainty. The possibility that a greater strength of human ODC promoter would result in an over-expression is not supported by our studies (H. Kuskinen, J. Wahlfors, L. Alhonen & J. Jänne, unpublished work) indi-

cating that the human promoter is substantially weaker than the mouse ODC promoter. A chromosomal position effect is in all likelihood ruled out by the very fact that out of seven consecutive transgenic mouse lines six lines over-expressed human ODC gene. The modestly prolonged half-life of ODC in the transgenic animals may marginally contribute to the enhanced enzyme activity, but certainly does not account for all of it. Methylation analyses performed with the isoschizomeric restriction endonucleases *HpaII* and *MspI* gave no indications of a tissue-specific hypomethylation of the transgene.

The most probable reason for the over-expression of human ODC gene in transgenic mice may be the gene construct used. Although the 800 nucleotides of the 5' flanking region of the gene confer full promoter activity of the gene (H. Kuisinen, J. Wahlfors, L. Alhonen & J. Jänne, unpublished work), it is not known whether there are more remotely located control elements primarily governing (tissue-specifically?) the gene activity. This idea is in fact supported by some recent experimental evidence. Katz & Kahana (1989) reported that, in association with the amplification of mouse ODC gene, a genomic rearrangement takes place, possibly deleting a putative silencer element for the gene. It is entirely possible that our gene construct used for the microinjections lacked such a regulatory element.

The reasons for the peculiar accumulation of putrescine in the testis and brain of the transgenic animals are not known, although the phenomenon may be related to the fact that these particular tissues have a blood/tissue barrier. Thus putrescine synthesized as a result of enhanced ODC activity in the transgenic animals may be sequestered in testis and brain because of the barrier not allowing a free exchange of the diamine. This view is in fact supported by our experiments indicating that intraperitoneally injected radioactive putrescine is taken up by mouse testis much less effectively than by liver.

In any event, the massive and preferential accumulation of putrescine in the testis of the transgenic animals is in accordance with the observed morphological and functional changes in this particular tissue (Halmekytö *et al.*, 1991). The fact that a striking over-expression of ODC in testis renders the male animals infertile may be a natural protection against spontaneous mutations leading to ODC overproduction. In any event, these transgenic mice offer a valuable model for studies aimed to elucidate the consequences of chronic overproduction of the polyamines.

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