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Research Article

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Patient Selection May Affect Gene Therapy Success

Dominant Negative Effects Observed for Ornithine Transcarbamylase in Mouse and Human Hepatocytes

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Abstract

We have achieved significant improvement of ornithine transcarbamylase deficiency (OTCD) in a mouse model through adenoviral-mediated gene transfer of the human ornithine transcarbamylase cDNA. Substantial reduction in orotic aciduria was observed within 24 h of treatment. Metabolic correction was later associated with phenotypic correction and moderate increase in enzymatic activity. In an effort to identify the level of gene expression required to achieve wild-type levels of enzyme activity we uncovered a dominant negative effect of the endogenous mutant protein on the activity of the delivered recombinant wild-type protein. This phenomenon is relevant to homomultimeric protein defects such as OTCD, which represent a challenging category of disorders for gene therapy. Thus, although our findings indicate that adenoviral-mediated gene transfer may have potential as a short-term treatment for OTCD in humans and may be effective especially during catabolic crisis, the observations in this study suggest that careful patient selection based on mutation class may be essential for initial OTCD gene therapy trials and, perhaps, for other homomultimeric enzyme deficiencies being considered as gene therapy targets. (*J. Clin. Invest.* 1996. 97:826–832.) **Key words:** ornithine transcarbamylase • liver • adenovirus • urea cycle • hyperammonemia

Introduction

Ornithine transcarbamylase (OTC)¹ deficiency (OTCD) is the most common and severe of the urea cycle disorders and is inherited as an X-linked recessive, with frequent new mutation occurrences (1, 2). Mutations range from deletions of the gene to point mutations that affect the activity and/or stability of the protein (1). The OTC protein is predominantly expressed in the liver and intestine with trace amounts found in kidney and brain (1). OTC is targeted to the mitochondria and assumes a homotrimer configuration in its active form (1). The deficiency is associated with citrulline and arginine depletion, orotic aciduria, hyperammonemia, and coma (3–6). Patients deteriorate rapidly if the hyperammonemia is not corrected within 24–48 h (2). Despite dietary and pharmacological intervention, the therapeutic outcome is inadequate and frequently associated with recurrent acute episodes resulting in mental retardation or, in the majority of cases, death (1–7). Liver transplantation is an option for qualified patients but can be associated with high morbidity and mortality (8, 9). Because of the severity of OTCD, the high frequency of new mutations, the limited organ target needed for enzymatic correction, and the currently inadequate therapy, we elected to develop a gene transfer method for treatment.

There are two mouse models for OTC deficiency: the sparse fur (*spf*) and the sparse fur–abnormal skin and hair (*spf^{ash}*) mutants. The molecular defect in the *spf* model is a missense point mutation in exon 4 which alters the pH optimum for the enzyme activity, resulting in only 20% wild-type activity at physiological pH despite high levels of expressed protein (10, 11). The *spf^{ash}* OTC gene has a splice junction mutation, leading to expression of 5–15% wild-type levels of active enzyme (12). The mutant protein subunits are imported into the mitochondria, but fail to form enzymatically active trimers (12, 13). The suitability of the OTCD mice as a model of the human disease has already been reported by Qureshi et al. (14). Before weaning, both mouse models are phenotypically small in size, display delayed development, and have wrinkled skin with little or no fur. Both models are characterized biochemically by reduced enzymatic activity and significant orotic aciduria (15–20-fold higher than normal). Orotic aciduria, which is one of the hallmarks of OTCD shared by mice and humans, is the most sensitive and persistent marker for OTCD in these animals (14, 15). Other findings associated with OTCD, such as hyperammonemia, low plasma citrulline, and depletion of arginine characteristic of OTCD patients, are not reliable diagnostic markers in these partially defective mouse models and

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1. *Abbreviations used in this paper:* Ad, adenoviral; CRM, cross-reacting material; OTC, ornithine transcarbamylase; OTCD, ornithine transcarbamylase deficiency; *spf*, sparse fur; *spf^{ash}*, sparse fur–abnormal skin and hair.

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furthermore are found to gradually normalize shortly after weaning (15).

Stratford-Perricaudet et al. (16) showed that adenoviral (Ad)-mediated transfer of the rat OTC cDNA resulted in partial correction of some of the *spf^{ash}* mice treated. We have successfully used replication-deficient E1-deleted E3-mutant Ad vectors encoding the human OTC cDNA (AdSR α hOTC) for vector-mediated transfer to hepatocytes in vitro (17). A similar vector encoding the β -galactosidase gene successfully expressed the marker gene in mouse liver in vivo (17). These data suggested that in vivo transfer and expression of human OTC could be achieved in mice.

In this report we describe our findings after in vivo gene delivery using the *spf^{ash}* mouse model. Reduction in orotic aciduria was rapid in onset (within 24 h) and was associated with phenotypic correction and increase in measurable enzymatic activity after a single injection of AdSR α hOTC. However, protein data suggest a dominant negative effect of this homotrimeric enzyme by coexpression of heterologous subunits, i.e., endogenous mutant (mouse or human) and human wild-type OTC.

Methods

Ad vectors. Construction of AdSR α hOTC and AdHCMVsp1LacZ recombinant vectors has been described previously (17). Both vectors were propagated and titered as described by Graham and Prevec (18). Briefly, the E1-deleted E3 mutant Ad5 vectors were grown on E1-complementing 293 cells (derived from human embryonic kidney cells) (19). The cells were inoculated at multiplicities of infection (moi's) of 10–20 plaque-forming units (pfu) per cell. When cytopathic effects were visible in 80–90% of the culture, at 36–48 h, cells were harvested, centrifuged, and pelleted. They were resuspended in 0.1 M Tris-HCl and frozen for storage at -70°C . The viral stock was prepared by repeated, 2–3, cycles of freeze-thaw in liquid nitrogen and 37°C water bath. Virus was purified and concentrated by two rounds of cesium chloride density centrifugation. Virus was dialyzed twice with 1 liter of suspension buffer (10 mM Tris, pH 7.5–8.0, 1 mM MgCl_2 , 10% glycerol). Titration was performed on 293 cells using a plaque-forming assay (18). Confluent (80–90%) 293 cells were grown overnight on 6-cm plates and were infected with serially diluted viral stock for 60 min, and overlaid with tissue culture medium containing 0.5% agarose. Plaques were counted at 6–8 d after infection.

Mouse colony. A homozygous/hemizygous breeding colony of *spf^{ash}* mice was maintained and propagated for use in this study. New-born pups (1–4 d old) were treated by direct liver injection of a single 10- μl aliquot of either the AdSR α hOTC, AdHCMVsp1LacZ, or suspension buffer. Direct injections into the liver parenchyma were performed, the liver was easily identified through the translucent skin of these very young animals. Injections were performed using a 33-gauge needle. Neither anesthetic nor surgical incision was required for this procedure. Pups were numbered by toe clipping to identify AdSR α hOTC-treated and control littermates. Urine was collected at successive time intervals and stored at -20°C until orotic acid levels were measured. Animals were killed by cervical dislocation and organs were removed for protein and DNA analysis. Animal care was in accordance with institutional guidelines.

DNA screening. Primers were designed to amplify a 700-bp target region spanning the SR α -hOTC junction. As an internal control, a 400-bp fragment of the mouse β -actin gene was coamplified using β -actin-specific primers. 2 μg of DNA extracted from the liver of killed animals was used for the PCR reaction following the standard recommended protocol (Perkin Elmer-Cetus, Norwalk, CT) in a final volume of 50 μl instead of 100 μl . 20 μl of the PCR product was run on a 1% agarose gel and PCR products were visualized by ethidium bromide staining followed by gel UV exposure.

Orotic acid assay. Orotic acid levels were measured in urine collected from AdSR α hOTC-treated *spf^{ash}* mice and controls (*spf^{ash}* mice treated with AdHCMVsp1LacZ or suspension buffer). Urinary orotic acid levels were measured according to Stajner and co-workers (20).

OTC enzyme assay. We used an established OTC assay for measurement of enzyme activity. Briefly, harvested cells were lysed in 0.25 M Tris-HCl, pH 7.2 or 9.5, and OTC activity in cell extract was measured using a radiochemical assay based on the conversion of [^{14}C]L-ornithine (Amersham Corp., Arlington Heights, IL) to [^{14}C]citrulline. Activity was measured based on the percentage of citrulline formed per milligram of total protein per hour of assay (21). Thin-layer chromatography was used to separate the radiolabeled reaction product citrulline from the substrate ornithine. Thin-layer chromatography plates were subjected to autoradiography, the spots were excised, and radioactivity was quantified by scintillation spectrophotometry.

Tissue cultures. Primary mouse hepatocyte cultures were prepared essentially as described by Grompe and co-workers (22). The 293 cell line, was maintained in MEM- α (GIBCO-BRL, Gaithersburg, MD) and the COS (transformed monkey kidney) cell line was maintained in Dulbecco's modified minimum essential medium (GIBCO-BRL). Both media were supplemented with heat-inactivated 10% newborn calf serum and 2 mM L-glutamine. Immediately before infection, cultures were washed three times in PBS and incubated in serum-free (COS) or serum-free hormonally defined (hepatocytes) media. Cells were infected at increasing moi's of AdSR α -hOTC vector and media were changed 2 h after infection to fresh serum-free media in which cultures were maintained until cells were collected.

Western blot analysis. Cells were collected 48–72 h after infection and total protein was extracted in RIPA and quantitated by the Bradford protein assay (23, 24). Duplicate samples of total protein were used for OTC enzyme assay and for Western blot analysis. Samples were run on SDS-denaturing polyacrylamide gels followed by transfer to nitrocellulose membranes. For the detection of human and mouse OTC protein, blots were incubated with a polyclonal antibody raised in rabbits against the carboxy-terminal epitope of the human OTC. The polyclonal antibody recognized both the human and the mouse OTC subunits as single bands at ~ 38 kD, with the human subunit migrating slower than the mouse. Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1:1,000 diluted; Sigma Immunochemicals, St. Louis, MO). The peroxidase on the blots was visualized using ECL chemiluminescence detection kit (Amersham Corp.) followed by autoradiography for 1–5 min. All blots were stripped and probed by a monoclonal antibody that recognizes β -actin (Sigma Immunochemicals) as an internal control.

Hybrid assay. Protein dissociation and reassociation conditions were essentially as described by Wentz and Schachman (25) using equimolar concentrations of mouse wild- and *spf*-mutant-type total protein extracts.

Statistical analysis. Statistical analysis was performed by Student's *t* test.

Results

Ad-mediated gene delivery in vivo. Titers equivalent to $1\text{--}5 \times 10^8$ pfu per gram weight were injected directly into the livers of 2–4-d-old OTCD mice. There were no significant differences in the survival rates of animals treated with this dosage of recombinant virus, compared with those treated with an equivalent dose of the control Ad vector expressing β -galactosidase or with suspension buffer. Livers of all treated animals were screened by PCR at the time of killing, confirming successful delivery and persistence of vector target sequence. The extent of biochemical correction was assessed by measurement of orotic acid levels in urine. In the OTCD mouse model, the in-

Table I. Levels of Orotic Acid in Urine after Treatment of the *spf^{ash}* Mice with the AdSR α hOTC Vector, Compared with Control Littermates, and the Percentage of Animals Showing Significant Reduction

Time after vector delivery	Orotic acid ($\mu\text{mol}/\text{mg}$ urinary creatinine)		Percent success	Percent orotic reduction
	AdSR α hOTC treated	Control		
24 h	25.5 \pm 9.7 (5)*	83.8 \pm 36.5 (4)	100%	70%
1 wk	13.4 \pm 2.5 (12) [‡]	20.8 \pm 7.3 (5)	80%	36%
2 wk	11.1 \pm 2.6 (10) [§]	27.2 \pm 12.2 (6)	100%	60%
3 wk	41.0 \pm 8.4 (6) [¶]	140.3 \pm 58.3 (4)	100%	71%

Significant differences were observed between treated animals and control littermates, $P < *0.02$, $^{\ddagger}0.05$, $^{\S}0.004$, and $^{\¶}0.01$. Wild-type orotic acid levels in urine of 3-wk-old mice were 16.3 \pm 4.8 μmol orotic acid per mg urinary creatinine ($n = 4$). Values represent mean \pm SEM. Number of animals is shown in parentheses.

crease in OTC levels with concomitant utilization of carbamyl phosphate has been shown to be directly reflected by urinary orotic acid excretion, providing a sensitive, noninvasive method for evaluating metabolic correction (14). Orotic acid levels over a 3-wk interval after treatment were compared with those of the control animals and demonstrated a therapeutic effect of AdSR α hOTC. Table I summarizes the results obtained from mice killed at 24 h, 1, 2, and 3 wk after treatment. Significant reduction in orotic acid levels was observed as early as 24 h after treatment and throughout the 3-wk period in these experiments.

Phenotypic correction characterized by rapid hair growth was observed in 42% of animals followed up to at least 2 wk after treatment in which there was an associated significant reduction in orotic acid levels compared with control littermates

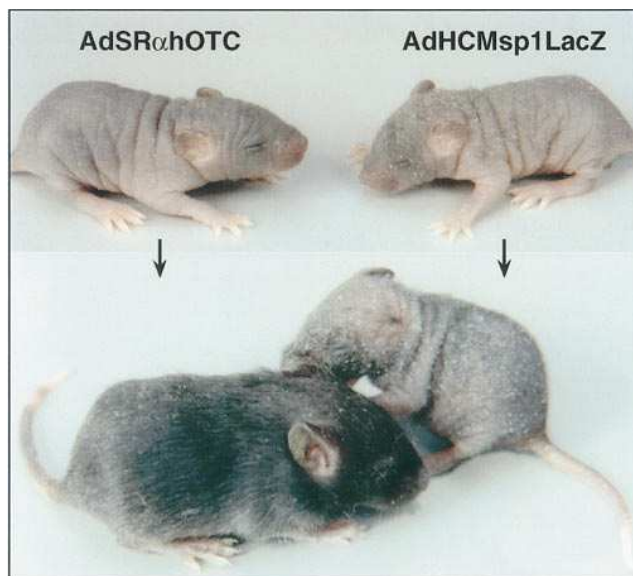


Figure 1. Ad-mediated in vivo correction of OTCD. Phenotypic difference in two littermates 2 wk after treatment. The mouse on the left (treated) and the one on the right (control) received injections directly into the liver with equivalent doses ($1\text{--}5 \times 10^8$ pfu per gram weight) of AdSR α hOTC and AdHCMVsp1LacZ virus, respectively.

(Fig. 1). Weight gain (23.7 \pm 8.3%) over control littermates was associated with the change in hair growth pattern.

Enzymatic activity was measured at 24 h and during the first, second, and third week, after treatment in both the liver and intestine (Fig. 2, A and B). Increase in enzyme activity was moderate and was observed in the intestine as early as 1 wk after treatment, using a standard OTC assay. At 3 wk, enzyme activity was ~ 1.3 - and 2.1-fold greater than basal levels in liver and intestine, respectively. We attribute the presence of re-

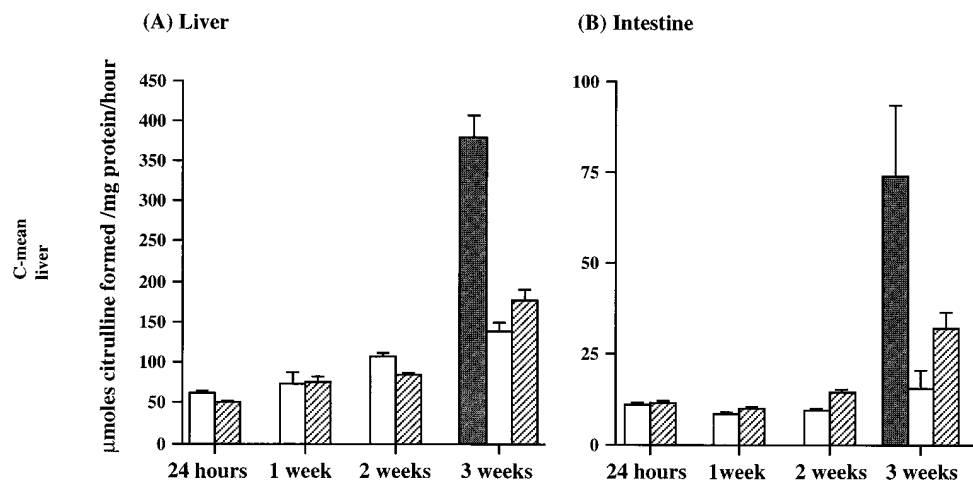


Figure 2. Enzyme activity measured in liver and intestine of AdSR α hOTC-treated (hatched bars) animals compared with controls (treated with AdHCMVsp1LacZ) and wild-type mice. Animals were killed at 24 h, 1, 2, and 3 wk after a single dose of viral delivery to *spf^{ash}* newborn mice (direct liver injection). OTC activity was measured in total protein extracts from liver (A) and intestine (B) of treated littermate control (open bars) and wild-type mice (filled bars). Activity was measured as micromoles of citrulline formed per milligram of total

protein per hour assay. In the liver an increase in enzyme activity (175.0 \pm 29.5 μmol citrulline formed/mg protein/h, $P \leq 0.03$) was observed at 3 wk in three out of five animals tested compared with control. Enzyme activity was ~ 1.3 -fold greater than basal levels (138 \pm 29.8). These levels were equivalent to 53.6% of enzyme activity measured in livers of wild-type mice ($n = 12$) matched by age (378.5 \pm 35.38). Enzyme activity levels in liver samples collected at earlier time points were not significantly different from control values. In the intestine, however, significant increase in enzyme activity over control values was measured for five out of nine (9.8 \pm 0.75 μmol citrulline formed/mg protein/h, $P \leq 0.04$, vs. 8.4 \pm 0.0), five out of five (14.3 \pm 1.95, $P \leq 0.04$, vs. 8.98 \pm 1.6), and four out of five (32.0 \pm 5.7, $P \leq 0.007$ vs. 15.5 \pm 6.3) animals at 1, 2, and 3 wk after treatment, respectively. At 3 wk, enzyme activity levels were 2.1-fold greater than basal levels and equivalent to 43.5% of activity levels (73.65 \pm 2.1 μmol citrulline/mg protein/h) measured from intestines of wild-type animals, matched by age ($n = 12$). Error bars represent SEM.

combinant adenovirus in the intestine to its access to the systemic circulation after the liver injections.

OTC expression and activity in primary mouse hepatocytes. The moderate increase in enzyme activity was associated with phenotypic correction and considerable reduction in orotic aciduria; however, measured OTC activity was significantly lower than wild-type activity levels. Although the data available thus far from the mouse experiments presented in this study and human asymptomatic patients with low levels of OTC activity are very encouraging regarding the prospect for gene replacement therapy in humans, we were interested in identifying the levels of protein expression needed to reach wild-type activity levels. Possible explanations for the moderate levels of activity detected include suboptimal promoter activity and thus gene expression, suboptimal treatment dosage, or limitations of the intrahepatic delivery method used. However, in this study the doses used were equivalent to or higher than those reported previously (16). Furthermore, in our hands several higher doses were examined but were found to be associated with toxic effects. In addition, in a limited set of experiments we examined intravenous (jugular) delivery and

did not observe an increase in enzymatic activity in liver or intestine. Therefore, we initiated studies to examine the levels of gene expression in vitro in an attempt to determine whether or not a stronger promoter is needed and to identify the level of protein expression required to achieve wild-type levels of OTC activity.

AdSR α hOTC was found to be highly efficient in mediating OTC gene expression in vitro as evident by the detected levels of transduced human OTC protein (Fig. 3). It was clear from AdSR α hOTC infection of COS, *spf*, and *spf^{ash}* cells that increasing multiplicity of the recombinant virus leads to a progressive increase in expressed human OTC protein, in all three infected cell cultures, as detected by Western blot analysis (Fig. 3 A). The band of cross-reacting material (CRM) detected in COS and mouse hepatocytes after infection with AdSR α hOTC migrated to identical positions in both cell types. The position of gel migration for human and rodent CRM represents the size of the cleaved precursor proteins. The ability to resolve the latter two bands by SDS-denaturing gel electrophoresis presumably reflects the 20-amino acid difference between the human and mouse proteins (excluding the

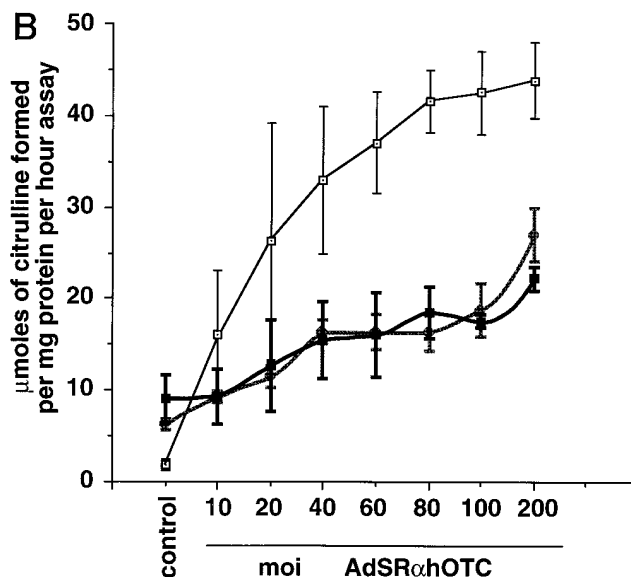
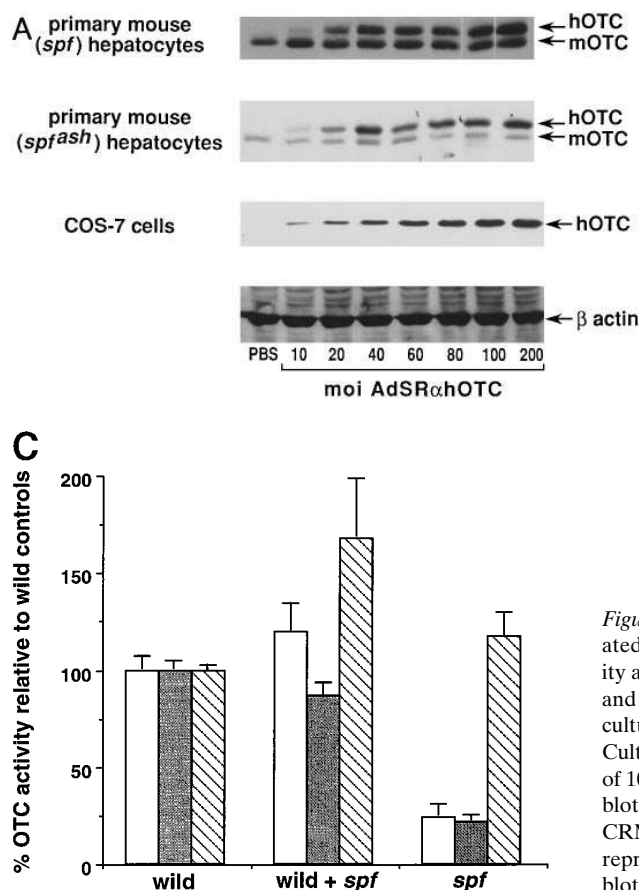


Figure 3. Ad-mediated human OTC protein expression. AdSR α hOTC-mediated human OTC protein expression was evaluated by measuring enzyme activity and detecting expressed protein by Western blot analysis. COS cells ($n = 3$) and primary mouse hepatocytes ($n = 4$) derived from *spf* and *spf^{ash}* livers were cultured and infected at moi's with the AdSR α hOTC vector 24 h after plating. Cultures were treated with PBS (mock infected) or with AdSR α hOTC at moi's of 10, 20, 40, 60, 80, 100, and 200. Total protein (25 μ g) was used for Western blot analysis (A) and for OTC enzyme assay (B). In A, hOTC represents the CRM of the human OTC protein expressed by the AdSR α hOTC and mOTC represents the CRM of the endogenously expressed mouse OTC protein. All blots were stripped and probed by a monoclonal antibody that recognizes β -actin (Sigma Immunochemicals) as an internal control. The bottom of A

shows equal levels of β -actin protein detected in the different COS samples. In B, activity was measured as micromoles of citrulline formed per milligram of total protein per hour assay. Significantly higher levels of OTC enzyme activity were measured in COS cells (open boxes) as compared with *spf* (gray circles) or *spf^{ash}* (filled boxes) hepatocytes infected at equivalent moi's (40, 60, 80, 100, and 200), error bars represent \pm SEM. C shows the difference in OTC activity detected in dissociated-reassociated mixtures (hybrid protein) of *spf* mutant and wild-type mouse protein subunits. Conditions were essentially as described by Wente and Schachman (21) using equimolar concentrations of mouse wild-type and *spf*-mutant type total protein extracts. OTC activity of mixed wild-type and *spf*-mutant protein was measured without dissociation and reassociation (native protein) at pH 7.2 (open bars) (119.5% \pm 13.85). Native control values were compared with hybrid protein activity measured at pH 7.2 (filled bars) (86.5% \pm 5.6) and pH 9.5 (hatched bars) (168% \pm 29.8) after hybrid formation. Activity of hybrid protein was reduced by 33% when compared with native equimolar mixture (119.5%) and 13.5% compared with unmixed wild-type ($n = 4$).

leader sequence). From these data it was clear that there was no apparent difficulty with the expression of the human OTC under the SR α promoter in the three cell types studied. However, when enzymatic activity was examined (Fig. 3 B), a different result was obtained. COS cells, which do not express endogenous OTC, rapidly increased OTC enzymatic levels commensurate with increasing protein levels. Only modest increases in OTC activity despite high levels of human protein were seen in both *spf* and *spf^{ash}* cells, which express mutant proteins. Such results strongly suggest interference with the human OTC activity. However, we could not be certain from these studies if the apparent dominant negative effect was mutation based or reflected structural differences in rodent and human OTC protein.

Given the X chromosome location of OTC and random X inactivation in females, heterotrimers of mutant and wild-type protein would not be expected to form. Gene transfer therefore provides the opportunity for such a phenomenon to occur in vivo that would not naturally occur. This concept was supported by in vitro data (Fig. 3 C), using the conditions of Wentz and Schachman, who successfully demonstrated the negative complementation of mutant and wild-type aspartate transcarbamylase subunits (25). Aspartate transcarbamylase shares common ancestry with OTC, displaying 50 and 75% homology at the primary and secondary structure levels, respectively (26). In our OTC studies, protein mixtures contained equimolar amounts of wild-type and *spf* mutant protein (which carries an OTC point mutation changing the pH optimum to pH 9.5). At physiological pH, 33% reduction in enzymatic activity was detected after dissociation-reassociation (hybrid formation) compared with control native mixtures which were assayed at the same pH and in which measured activity was equal to the expected cumulative levels. The reduction in net activity as determined by these in vitro studies is in agreement with the in vitro cellular results. This finding further supports impairment of wild-type activity presumably in the presence of heterotrimers. Collectively, the data suggest that OTC enzyme activity correction may be underestimated in the mouse model where both mutant protein and human correction genes are coexpressed.

OTC expression and activity in primary human hepatocytes. The above observations may be a significant consideration for human trials. Therefore, we elected to clarify the issue by study of gene transfer in human OTC liver cells. Liver transplantation is a corrective therapy for OTC deficiencies and such rare operative cases serve as a source of liver tissue. We have studied two patients with ideal mutant variation. One patient had a missense *Taq* site mutation in exon 3, which did not affect protein expression, as determined by Western blot analysis, however, it completely abolished the enzymatic activity (Fig. 4, A and B). The second patient had undetectable levels of both protein and enzymatic activity as shown in Fig. 4, C and D (molecular bases of mutation are currently under investigation). Thus, the liver sample from the former patient was CRM negative, whereas the sample obtained from the latter patient was CRM positive. Primary hepatocyte cultures were established from both livers as well as COS cell cultures and following the identical protocol used for the primary mouse hepatocytes, cultures were infected with AdSR α hOTC at increasing moi's, collected, and compared for recombinant human OTC protein expression and corresponding enzymatic activity. Similar to our finding in the mouse hepatocyte experiments,

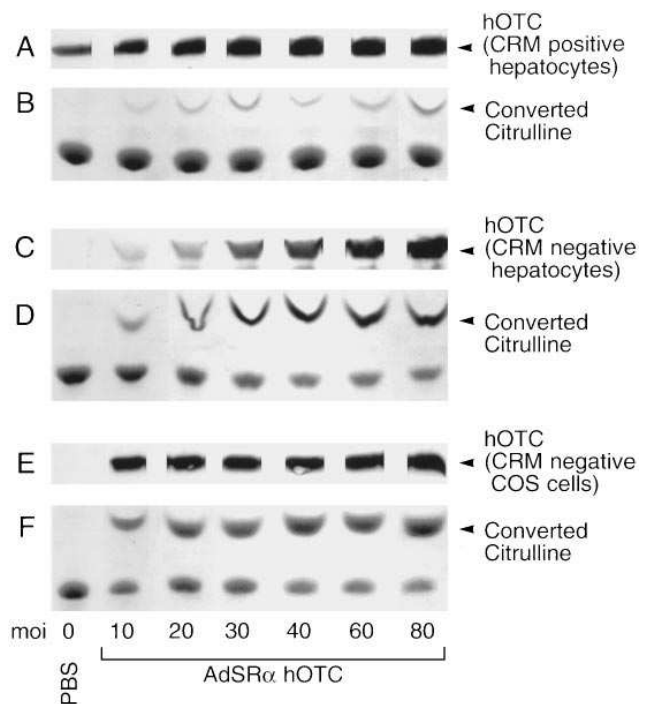


Figure 4. Ad-mediated hOTC protein expression in primary human hepatocytes. Identical to the primary mouse experiments, AdSR α -hOTC-mediated human OTC protein expression in primary human hepatocytes derived from livers of OTCD patients was evaluated. COS cells and the primary human OTCD hepatocytes (CRM positive and CRM negative) were cultured and infected with PBS (mock infected) or with AdSR α hOTC at moi's of 10, 20, 30, 40, 60, and 80, 24 h after plating. Media were changed 4 h after infection. Cultures were maintained on serum free (COS) or serum-free hormonally defined (hepatocytes) media. Cells were collected 48–72 h after infection when total protein was extracted in RIPA and quantitated by the Bradford protein assay. Duplicate samples were used for Western blot analysis (A, C, and E) and for OTC enzyme assay (B, D, and F). The results of the corresponding samples measured for OTC activity are shown on autoradiographs of the TLC plates which were used to separate the citrulline (arrowhead) from the substrate ornithine of the OTC enzyme reaction (B, D, and F). OTC activity was measured and compared between samples and among the three sets of cultures.

enzymatic activity measured after AdSR α hOTC-mediated OTC protein expression in the CRM-positive hepatocytes was substantially inhibited in comparison to COS cells (Fig. 4, B and F). In contrast, activity measured for the AdSR α hOTC-mediated OTC protein expressed in the CRM-negative hepatocytes increased in parallel to the increased protein expression detected by Western blot analysis (Fig. 4, C and D) and in agreement with results obtained for the infected COS cells which, similarly, do not express endogenous mutant OTC protein (Fig. 4, E and F). Specific activity of the recombinant human OTC protein expressed in the CRM-negative human hepatocytes was at least 50-fold higher than that measured for the protein detected in the treated CRM-positive human hepatocytes (Table II).

Discussion

This study describes our findings using an Ad vector carrying the human OTC cDNA in correcting the OTCD mouse model.

Table II. OTC Activity Expressed per Unit Immunoreactive Protein of the Control and AdSR α OTC-infected Primary Human Hepatocytes and COS Cell Cultures

	Specific activity	Ratio relative to COS
Patient 1 (CRM negative)	28.02 \pm 7.17 (8)	2.80
COS (CRM negative)	9.99 \pm 1.16 (8)	
Patient 2 (CRM positive)	0.56 \pm 0.04 (8)	0.06
COS (CRM negative)	9.99 \pm 1.07 (8)	

Relative specific activity is given as arbitrary units which represent OTC activity divided by the intensity of the corresponding CRM band determined by scanning densitometry (28). In case of the CRM-positive samples, intensity values measured for the control samples were subtracted from the intensity of the AdSR α OTC-infected samples before determining the specific activity value. The mean values \pm SE and the number of samples (in parentheses) are given.

Phenotypic improvement was achieved and reduction in orotic acid levels was detected within 24 h of gene delivery. Given the need for a new therapy for OTCD in humans for the acute hyperammonemic episodes of these patients, Ad-mediated OTC delivery and expression may be useful in a crisis setting. The biochemical results revealed that only moderate increases in enzyme activity had led to in vivo metabolic and phenotypic correction, and are consistent with human studies which indicate that low levels of OTC ameliorate the phenotype. Indeed, Matsuda et al. (27) have shown that OTCD patients with \sim 5% of normal enzyme activity have only mild phenotypes. The increase in the levels of activity detected in the intestine at early time points may have contributed to the observed rapid reduction in orotic acid levels in urine. Our previous transgenic studies have documented the importance of small bowel expression (21). In vitro, Ad-mediated OTC protein expression and activity were detected as early as 18 h after treatment. An intriguing finding in our studies was the unexpected low measurable increase in enzymatic activity and the clear disparity between OTC protein levels and corresponding activity (Figs. 3 and 4). Given the homotrimer active form of the OTC enzyme, the protein data collected suggest that mutant subunits could interfere with the resulting activity of the wild-type vector-delivered protein. Our correcting transgenic studies of *spf* mice achieved remarkable enzymatic activity exclusively in small bowel which has 1/5 the level of endogenous mutant OTC compared with the liver and perhaps less heterotrimer formation (17). In addition, it is interesting that in our studies using retroviral-mediated transfer of the human OTC cDNA to OTCD-*spf* primary mouse hepatocytes increases in OTC activity were higher at 15 d after infection than at 8 d (22). Of interest, this coincided with the marked reduction in endogenous OTC activity observed in primary hepatocytes at 2 wk after culture and was observed only at that delayed time point despite the fact that in these studies the retroviral-mediated wild-type mRNA levels had reached a plateau as early as day 4 after infection and that the half-life of OTC is \sim 6–9 d (22). These results further support our speculations that the moderate correction of the CRM-positive OTCD mouse by human cDNA underestimates the potential corrective power in the more severe CRM-negative subpopulation of OTCD patients. Thus, the selection of patients may be critical to the evaluation of a gene transfer method when multimeric subunits constitute

the active gene product. One may speculate that the use of a stronger promoter and overexpression of OTC may overcome this potential limitation; however, OTC is a stable protein (6–9 d), and although high levels of protein expression may be effective initially, the effects of accumulating this mitochondria-targeted protein for such long periods will have to be carefully evaluated. OTCD is problematic in this respect since the mutations are frequently de novo, heterogeneous, and result in differing OTC enzyme/protein levels and, thus, phenotypically patients range from asymptomatic or mild to severely affected. Therefore, this study represents the first report demonstrating potential dominant negative effects as a consequence of a gene therapy attempt for homomultimeric protein defects; while focus was on OTC correction, the results found likely apply to other multimeric gene defects targeted for genetic correction.

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References

1. Brusilow, S.W., and A.L. Horwich. 1989. The Metabolic Basis of Inherited Disease. 7th edition. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, editors. McGraw-Hill, New York, NY. 1187–1232.
2. Maestri, N., E. Hauser, D. Bartholomew, and S. Brusilow. 1991. Prospective treatment of urea cycle disorder. *J. Pediatr.* 119:923–928.
3. Arn, P.H., E.R. Hauser, G.H. Thomas, G. Herman, D. Hess, and S.W. Brusilow. 1990. Hyperammonemia in women with a mutation in the ornithine transcarbamylase locus: a cause of postpartum coma. *N. Engl. J. Med.* 322:1652–1655.
4. Rowe, P.C., S.L. Newman, and S.W. Brusilow. 1986. Natural history of symptomatic partial ornithine transcarbamylase deficiency. *N. Engl. J. Med.* 314:541–547.
5. Batshaw, M., M. Msall, A. Beaudet, and J. Trojak. 1986. Risk of serious illness in heterozygotes for ornithine transcarbamylase deficiency. *J. Pediatr.* 108:236–241.
6. Finkelstein, J.E., C.A. Francomano, S.W. Brusilow, and M.D. Traystman. 1990. Use of denaturing gradient gel electrophoresis for detection of mutation and prospective diagnosis in late onset ornithine transcarbamylase deficiency. *Genomics.* 7:167–172.
7. Brusilow, S.W. 1991. Treatment of Urea Cycle Disorders. R.J. Desnick, editor. Churchill-Livingstone, Inc., Naperville, IL. 1–79.
8. Largilliere, C., D. Houssin, F. Gottrand, C. Mathey, A. Checoury, D. Alagille, and J.-P. Farriaux. 1989. Liver transplantation for ornithine transcarbamylase deficiency in a girl. *J. Pediatr.* 115:415–417.
9. Goldstein, R.M., G.K. Klintmalm, B.S. Husberg, T.A. Gonwa, and D. Polter. 1991. Liver transplantation: a review of the first 200 patients. *Transplant. Proc.* 23:1780–1781.
10. DeMars, R., S.L. LeVan, B.L. Trend, and L.B. Russell. 1976. Abnormal ornithine carbamoyltransferase in mice having the sparse-fur mutation. *Proc. Natl. Acad. Sci. USA.* 73:1693–1697.
11. Briand, P., L. Cathelineau, P. Kamoun, D. Gigot, and M. Penninckx. 1981. Increase in ornithine transcarbamylase protein in sparse-fur mice with ornithine transcarbamylase deficiency. *FEBS Lett.* 130:65–68.
12. Doolittle, D.P., L.L. Hulbert, and C. Cordy. 1974. A new allele of the sparse fur gene in the mouse. *J. Hered.* 65:194–195.
13. Hodges, P.E., and L.E. Rosenberg. 1989. The *spf^{ash}* mouse: a missense mutation in the ornithine transcarbamylase gene also causes aberrant mRNA splicing. *Proc. Natl. Acad. Sci. USA.* 86:4142–4146.
14. Quershi, I.A., J. Letarte, and R. Ouellet. 1979. Ornithine transcarbamylase deficiency in mutant mice. I. Studies on the characterization of enzyme defect and suitability as animal model of human disease. *Pediatr. Res.* 13:807–811.
15. Gushiken, T., N. Yoshimura, and T. Saheki. 1985. Transient hyperammonemia during aging in ornithine transcarbamylase-deficient, sparse-fur mice.

Biochem. Int. 11:637-643.

16. Stratford-Perricaudet, L.D., M. Levrero, J.-F. Chasse, M. Perricaudet, and P. Briand. 1990. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Human Gene Ther.* 1: 241-256.

17. Morsy, M.A., E.L. Alford, A. Bett, F.L. Graham, and C.T. Caskey. 1993. Efficient adenoviral-mediated OTC expression in deficient mouse and human hepatocytes. *J. Clin. Invest.* 92:1580-1586.

18. Graham, F.L., and L. Prevec. 1991. Methods in Molecular Biology: Gene Transfer and Expression. The Humana Press Inc., Clinton, NJ, 109-128.

19. Graham, F.L., J. Smiley, W.C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus 5. *J. Gen. Virol.* 36:59-74.

20. Stajner, A., J. Suva, and F. Musil. 1968. The determination of orotic acid in the blood serum by means of the spectrophotometric method. *Experientia (Basel)*. 24:116-117.

21. Jones, S.N., M. Grompe, M.I. Munir, G. Veres, W.J. Craigen, and C.T. Caskey. 1990. Ectopic correction of ornithine transcarbamylase deficiency in sparse fur mice. *J. Biol. Chem.* 265:14684-14690.

22. Grompe, M., S.N. Jones, H. Loulseged, and C.T. Caskey. 1992. Retroviral-mediated gene transfer of human ornithine transcarbamylase into primary

hepatocytes of *spf* and *spf-ash* mice. *Hum. Gene Ther.* 3:35-44.

23. Antibodies: A Laboratory Manual. 1988. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

24. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

25. Wente, S.R., and H.K. Schachman. 1987. Shared active sites in oligomeric enzymes: model studies with defective mutants of aspartate transcarbamoylase produced by site directed mutagenesis. *Proc. Natl. Acad. Sci. USA.* 84: 31-35.

26. Volz, K.W., K.L. Krause, and W. Lipscomb. 1986. The binding of *N*-(phosphonacetyl)-*L*-aspartate to aspartate carbamoyltransferase of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 136:822-826.

27. Matsuda, I., N. Nagata, T. Matsuura, K. Oyanagi, K. Tada, K. Narisawa, T. Kitagawa, T. Sakiyama, F. Yamashita, and M. Yoshino. 1991. Retrospective survey of urea cycle disorders. Part 1. Clinical and laboratory observations of thirty-two Japanese male patients with ornithine transcarbamylase deficiency. *Am. J. Med. Genet.* 38:85-89.

28. Gruppuso, P.A., and J.M. Boylan. 1995. Developmental changes in the activity and cellular localization of hepatic casein kinase II in the rat. *J. Cell. Biochem.* 58:65-72.