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AMELIORATION OF PROLIDASE DEFICIENCY IN FIBROBLASTS USING ADENOVIRUS MEDIATED GENE TRANSFER

Kumiko IKEDA,[1,2] Jun TOHYAMA,[1] Seiichi TSUJINO,[1] Kahei SATO,[2] Takashi OONO,[3] Jirô ARATA,[3] Fumio ENDO,[4] and Norio SAKURAGAWA[1,*]

[1] *Department of Inherited Metabolic Disease, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan*
[2] *Department of Applied Biological Science, College of Agriculture and Veterinary Medicine, Nihon University, 1866 Kameino, Fujisawa 252, Japan*
[3] *Department of Dermatology, Okayama University Medical School, 2-5-1 Shikatacho, Okayama 700, Japan*
[4] *Department of Pediatrics, Kumamoto University Medical School, 1-1-1 Honjo, Kumamoto 860, Japan*

Summary Prolidase deficiency is an autosomal recessive inherited disease characterized clinically by frequent infections, mental retardation, and various skin lesions. Fundamental treatments for these manifestations have not been established. We performed adenovirus-mediated gene transfer of human prolidase cDNA into fibroblasts from patients with prolidase deficiency. Infection with the adenovirus vector carrying human prolidase cDNA increased prolidase activity in fibroblasts up to approximately 7.5 times of that of normal control fibroblasts. This indicates the feasibility of adenovirus-mediated gene therapy to treat patients with prolidase deficiency in the future.

Key Words prolidase deficiency, adenovirus vector, gene therapy

INTRODUCTION

Prolidase (peptidase D, iminopeptidase, EC 3.4.13.9) is an ubiquitous oligopeptidase present in mammals which releases carboxyterminal proline or hydroxyproline from oligopeptides. The human enzyme has been isolated, showing a mono-dimer with a subunit of 54,200 daltons (Endo *et al.*, 1987, 1988). This enzyme gene (gene symbol, PEPD), located on chromosome 19p13.2, is over 130

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* To whom correspondence should be addressed.

kb long and is split into 15 exons (Endo *et al.*, 1989; Tanoue *et al.*, 1990). The subunit protein is composed of 492 amino acid residues and is rich in glutamic acid residues (Endo *et al.*, 1989). Several genetic alterations in the prolidase gene associated with prolidase deficiency have been described (Tanoue *et al.*, 1990, 1991; Ledoux *et al.*, 1996).

Clinically, prolidase deficiency is characterized by mental retardation, various skin lesions, splenomegaly, and abnormalities of collagenous tissues (Phang and Scriver, 1995). Many therapeutic trials have been conducted including dietary supplements and daily applications of topical ointment. In addition, transfusion with normal erythrocytes and skin grafts have been performed. Although some improvements in skin lesions have been reported, a search for more fundamental treatments for other putative manifestations of prolidase deficiency has been carried out.

In the present study, we carried out adenovirus-mediated transfer of human prolidase cDNA into fibroblasts from patients with prolidase deficiency. We discussed the feasibility of *ex vivo* gene therapy for prolidase deficiency on the basis of amelioration of the prolidase deficiency in fibroblasts as shown by this experiment.

MATERIALS AND METHODS

Cell lines. Cultured skin fibroblasts were obtained from two Japanese female siblings with prolidase deficiency. Clinical features (Arata *et al.*, 1979) and a molecular defect in these patients have been described previously (Tanoue *et al.*, 1990). Normal and affected human skin fibroblasts and human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, penicillin, and streptomycin at 37°C in 5% CO_2.

Construction of recombinant adenovirus. Recombinant adenovirus containing the PEPD cDNA was generated by the COS-TPC method (Miyake *et al.*, 1996). The PEPD cDNA was filled with the Klenow enzyme and inserted into the *Swa*I site of cosmid pAx1CAwt (Miyake *et al.*, 1996). The cosmid DNA was mixed with the *Nsi*I-digested DNA-terminal protein complex of Ad5-dIX (Saito *et al.*, 1985) and 293 cells were transfected with the mixed DNA by the calcium-phosphate method using the CellPhect Transfection Kit (Pharmacia). A recombinant adenovirus AxCAPEPD was generated through homologous recombination in 293 cells.

Gene transduction utilizing recombinant adenovirus. Cells were exposed to recombinant adenovirus in a minimal volume at the desired multiplicity of infection (MOI) in DMEM containing 10% FCS and left at 37°C in 5% CO_2 for 1 hr. After the addition of DMEM containing 10% FCS, the cells were left for 3 days.

X-gal staining. Recombinant adenovirus containing β-galactosidase gene

(AxCALacZ) was obtained from Dr. Saito (The University of Tokyo). Cultured skin fibroblasts were transfected with AxCALacZ at a MOI 1, 20 and 50. Three days after infection the cells were washed with PBS(−) twice and fixed with 1% formaldehyde. After washing with PBS(−), the cells were stained for 4 hr at 37°C with X-gal reaction mixture containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, 4 mg/ml X-gal (Euromedex) dissolved in *N,N*-dimethyl formamide. The slides were rinsed with PBS(−), air dried and mounted.

Northern blot analysis. Cultured skin fibroblasts were infected with AxCAPEPD at MOI 20. The cells were harvested 3 days after infection. Total RNA was extracted by AGPC methods. Twenty micrograms of total RNA was separated on a 1.0% formaldehyde agarose gel and transferred to a nylone membrane and hybridized at 68°C overnight. The RNA probe was derived from the PEPD cDNA and labeled with digoxigenin-UTP using DIG RNA Labeling Kit (Boehringer). Specific RNA was detected by autoradiography with chemiluminescence of CDP (TROPIX) as described (Engler-Blum *et al.*, 1993).

Prolidase assay. AxCAPEPD was transfected into cultured skin fibroblasts from patients at MOI 20. The cells were harvested 3 days after infection, and the prolidase activity was measured by a modification of the method described previously (Endo and Matsuda, 1981). The cells were suspended in 0.1 M Tris-HCl (pH 7.4) and sonicated. Then centrifuged at $12,000 \times g$ for 3 min. Supernatant (50 μl) was mixed with 75 μl of 0.1 M $MnCl_2$, 25 μl of 0.1 M Tris-HCl (pH 7.4), 50 μl of distilled water and then was preincubated at 37°C for 1 hr to obtain maximal enzyme activity. Fifty microliters of 0.1 M Gly-Pro (SIGMA) was added, followed by incubation at 37°C for 15 min. The reaction was stopped by the addition of 250 μl of a 10% trichloroacetic acid (TCA) solution and the supernatant was used for proline colorimetric determination by the methods of Cinard (1952). Protein was measured by the method of Lowry *et al.* (1951).

RESULTS

Recombinant adenovirus vector

Forty-seven virus clones were obtained in this experiment. Eight of 47 virus clones were assessed by restriction analysis, and 4 of these clones were identified as expected virus clones. The structure of AxCAPEPD was shown in Fig. 1. AxCAPEPD includes CAG promotor (Niwa *et al.*, 1991), polyadenylation sequence, prolidase (PEPD) cDNA. The titer of the viral clone used for infection of fibroblasts was 5.94×10^9 pfu/ml.

Induction rate estimated by X-gal staining

Cultured fibroblasts were infected with AxCALacZ. Although the cells were infected at MOI 1, 20, and 50, infection at MOI 20 resulted in the most efficient

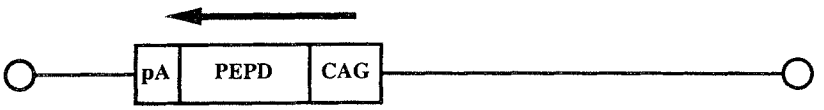

Fig. 1. Structure of the recombinant adenovirus AxCAPEPD. CAG, CAG promoter; PEPD, prolidase cDNA; pA, rabbit β-globin polyadenylation sequence. Open circles at the both end indicate Ad5 terminal proteins. The arrow shows the orientation of the transcription.

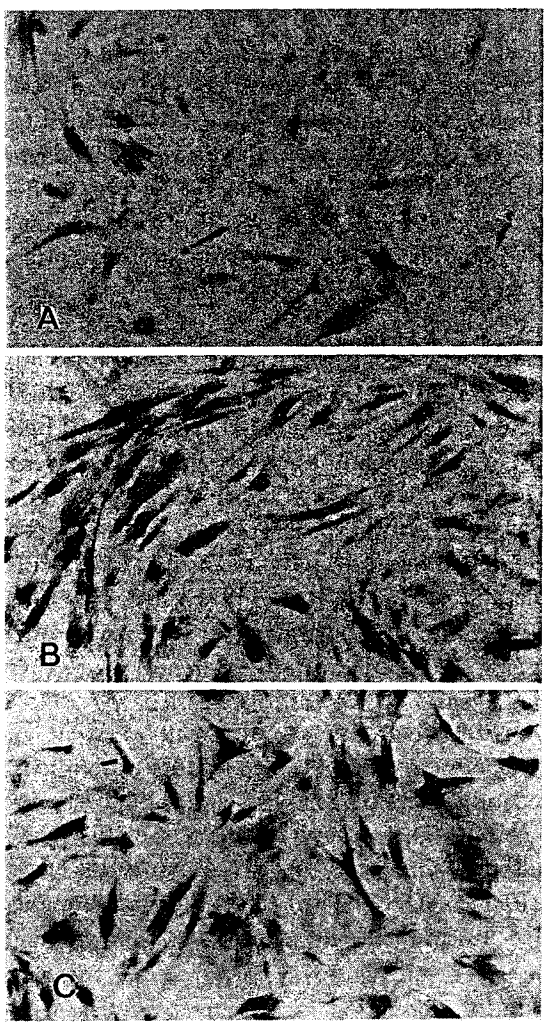

Fig. 2. X-gal staining of cultured fibroblasts infected with AxCALacZ at MOI (A) 1, (B) 20, (C) 50.

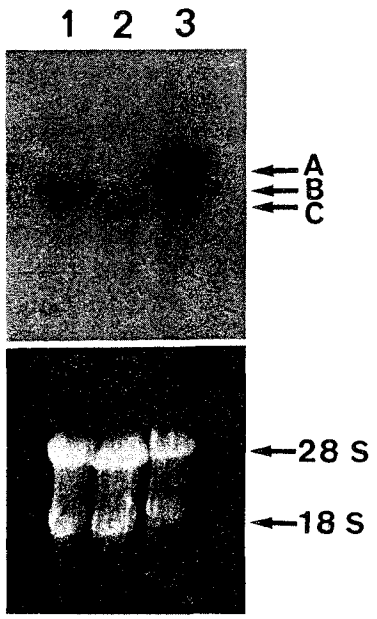

Fig. 3. Northern blot analysis of PEPD mRNA. Lane 1, normal fibroblasts; lane 2, prolidase-deficient fibroblasts; lane 3, prolidase-deficient fibroblasts infected with AxCAPEPD. A, PEPD RNA expressed by the infection with AxCAPEPD; B, normal PEPD RNA; C, abnormal PEPD RNA from fibroblasts of the patient. Shown below the blot is ethidium bromide-stained RNA before transfer.

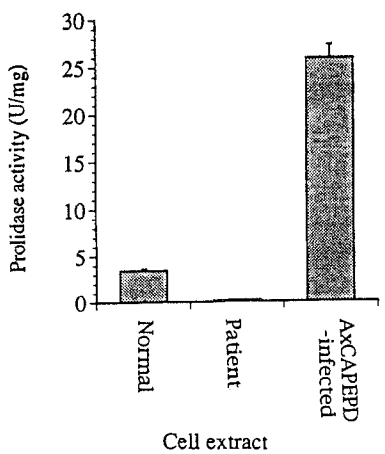

Fig. 4. Prolidase activity of normal fibroblasts, prolidase-deficient fibroblasts, and prolidase-deficient fibroblasts infected with AxCAPEPD. Error bars refer to standard error.

induction (almost 100%) (Fig. 2).

Northern blot analysis

The cultured fibroblasts were infected with AxCAPEPD. Northern blot analysis showed that abnormal PEPD mRNA from the patient's fibroblasts was shorter than normal mRNA because of the deletion of exon 14. There were two bands in the lane of infected cells: the shorter one corresponded to endogenous abnormal prolidase mRNA, and the longer one, which was thicker, indicated the transferred PEPD mRNA (Fig. 3). The longer transferred PEPD mRNA was obtained because the rabbit β-globin polyadenylation sequence was included in order to construct AxCAPEPD.

Correction of the prolidase deficiency in fibroblasts from patients

The primary fibroblasts from a patient with prolidase deficiency lacked prolidase activity (0.28–0.08 U/mg) as compared to normal control cells (3.44 ± 0.2 U/mg). To correct the prolidase activity of affected fibroblasts, we infected the cells with AxCAPEPD (MOI 20), which induced the prolidase level up to approximately 7.5 times higher than that of the control cells (Fig. 4).

DISCUSSION

Prolidase deficiency has a clinical phenotype of highly variable expressivity, varying among affected persons from no manifestations to severe progressive skin ulceration, decreased resistance to infection, and impaired cognitive development. The skin disease can be devastatingly severe and impaired motor or cognitive development is the second most important manifestation of prolidase deficiency (Phang and Scriver, 1995). No effective treatment for prolidase deficiency has been reported, although several therapeutic trials have been described. Transfusion of normal erythrocytes containing prolidase activity has raised endogenous prolidase activity (Endo *et al.*, 1982) but without an effect on skin signs or imidodipeptiduria (Ogata *et al.*, 1981; Isemura *et al.*, 1981). Skin grafts failed to cure the lesions (Ogata *et al.*, 1981), but daily applications of topical L-proline and glycine have improved leg ulcers (Arata *et al.*, 1986). These observations, together with the recent advances in gene transfer, prompted us to evaluate the possibility of gene therapy for prolidase deficiency.

We have constructed adenovirus vectors, carrying the human prolidase cDNA (AxCAPEPD). Judging from the X-gal staining where almost 100% of the cells infected with AxCALacZ were blue-stained, the induction efficiency indicated that the adenovirus vector was an appropriate tool for gene transfer to fibroblasts. The Northern blot analysis showed the larger amount of production of PEPD mRNA, indicating a strong gene expression. In this study the gene transfer using adenovirus vectors increased prolidase activity in fibroblasts from the patients up to

approximately 7.5 times higher than that of normal fibroblasts. This suggests that the recombinant adenovirus containing the PEPDcDNA should be effective in *ex vivo* gene therapy of fibroblasts.

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