Restoration of arylsulphatase B activity in human mucopolysaccharidosis-type-VI fibroblasts by retroviral-vector-mediated gene transfer

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The Maroteaux–Lamy syndrome (mucopolysaccharidosis type VI; MPS VI) is a lysosomal storage disease caused by deficiency of the enzyme arylsulphatase B (ASB). A human ASB cDNA has been subcloned into the retroviral vector pXT1 containing the bacterial neomycin-resistance gene and an internal thymidine kinase promoter for transcription of the inserted gene. Replication defective retrovirus was generated by transfecting the construct into the amphotropic packaging cell line PA317. Human MPS VI fibroblasts infected with recombinant retrovirus integrated the provirus into their genome and expressed retrovirus-encoded ASB mRNAs. In infected fibroblasts the level of ASB was up to 36-fold higher than in normal fibroblasts. Biosynthesis and processing of ASB in infected MPS VI fibroblasts was accomplished as in normal fibroblasts, and mature, enzymically active, ASB accumulated in dense lysosomes, indicating that the ASB deficiency in MPS VI fibroblasts was corrected by the retroviral gene transfer.

INTRODUCTION

Mucopolysaccharidosis type VI (MPS VI) is a glycosaminoglycan storage disorder with autosomal recessive inheritance resulting from a deficiency of the lysosomal enzyme arylsulphatase B (ASB; EC 3.1.6.1). ASB catalyses the hydrolysis of the sulphate ester group from *N*-acetylgalactosamine 4-sulphate residues of dermatan sulphate (Matalon *et al.*, 1974; O'Brien *et al.*, 1974). Deficiency of ASB activity leads to intralysosomal accumulation and urinary excretion of partially degraded dermatan sulphate. MPS VI patients exhibit a variety of clinical phenotypes. Patients with the severe form of the disease present corneal clouding, hepatosplenomegaly, skeletal malformation and growth retardation, whereas the mental development is normal or near-normal in all MPS VI forms. Most patients with severe MPS VI die of heart failure in the second or third decade [for a review, see Neufeld & Muenzer (1989)].

Currently there is no specific treatment of mucopolysaccharidoses available. Attempts to replace the deficient lysosomal enzymes by infusion of purified enzyme, plasma or leucocytes yielded, if at all, only transient improvement. The limited amount of enzymes infused and the lack of functional recognition markers necessary for receptor-mediated endocytosis of the infused lysosomal enzymes may explain the disappointing results of these therapeutic trials. As an alternative approach allogeneic bone-marrow transplantation was pursued in several mucopolysaccharidoses under the assumption that haematopoietic progenitor cells (e.g. tissue macrophages) will secrete lysosomal enzymes, which can be taken up by the host cells and restore the deficient enzyme activity. A bone-marrow transplantation in a patient with severe MPS VI resulted in an increase of ASB activity in peripheral lymphocytes and granulocytes, a decrease in urinary glycosaminoglycan excretion and substantial reduction of the hepatosplenomegaly, but no improvement of visual and skeletal involvement was achieved (Krivit *et al.*, 1984). Allogeneic bone-marrow transplantation, even when HLA (histocompatibility antigens)-identical donors are available, carries a

high risk of mortality and morbidity from graft-versus-host disease (Neufeld & Muenzer, 1989).

With the development of replication-defective retroviral vectors capable of efficient transfer and expression of genes in a variety of mammalian cells, approaches to somatic-cell gene therapy for certain genetic disorders has become feasible (Anderson, 1984; Keller *et al.*, 1985; Temin, 1986). Hitherto these approaches have focused on gene transfer into haematopoietic stem cells *in vitro* and subsequent implantation of the manipulated cells into recipient animals. In contrast with the murine system, where stable levels of expression in cells derived from the haematopoietic system *in vivo* have been reported (Eglitis *et al.*, 1985; Keller *et al.*, 1985; Dzierzak *et al.*, 1987), this approach has been seriously hampered in dogs and monkeys, owing to poor infection of haematopoietic stem cells *in vitro* and rapidly declining expression *in vivo* (Williams *et al.*, 1986; Kantoff *et al.*, 1987; McIvor *et al.*, 1987; Stead *et al.*, 1988). Skin fibroblasts provide an alternative target for gene therapy, since they can be efficiently infected with retroviruses and subsequently selected and expanded in culture (Palmer *et al.*, 1987; Miyanojara *et al.*, 1988; Osborne & Miller, 1988). Furthermore, it has been shown that retrovirus-infected mouse fibroblasts that expressed a foreign gene coding for a secretory protein *in vitro* continued to express stable amounts of this gene product *in vivo* even in the absence of selective pressure (Garver *et al.*, 1987).

MPS VI appears to be an appropriate model for gene therapy approaches of lysosomal storage diseases, since ASB cDNAs have been isolated recently (Peters *et al.*, 1990; Schuchman *et al.*, 1990). Furthermore, a feline model of the disease has been described (Jezyk *et al.*, 1977), and a successful allogeneic bone-marrow transplantation in an affected cat resulting in encouraging improvements of the symptoms has been reported (Gasper *et al.*, 1984).

Here we report insertion of a human ASB cDNA into a retroviral vector and efficient transfer of the gene into human MPS VI fibroblasts, resulting in high levels of expression of ASB enzyme activity *in vitro*. Furthermore, normal processing and lysosomal localization of the expressed ASB are shown.

Abbreviations used: MPS VI, mucopolysaccharidosis type VI (Maroteaux–Lamy syndrome); ASB, arylsulphatase B; TK, thymidine kinase; LTR, long terminal repeat; G418, geneticin; *neo*^R, neomycin-resistance gene; poly(A)⁺, polyadenylated.

MATERIALS AND METHODS

Cell lines and vectors

Mutant cell line 05343S (5343) was obtained from The Human Genetic Cell Repository (Lyon, France). The packaging cell line PA317 was kindly provided by K. Pfizenmeyer (Max-Planck-Institut, Göttingen, Germany) and the retroviral vector pXT1 by E. F. Wagner (Institute for Molecular Pathology, Vienna, Austria).

Production of recombinant retrovirus XT-ASB

The 1903 bp *XhoI*-*SalI* DNA fragment of plasmid pCA7 encoding the first 29 amino acids of the signal peptide of lysosomal acid phosphatase and amino acids 37–533 of ASB (Peters *et al.*, 1990) was ligated into the *XhoI* site of the retroviral expression vector pXT1 (Boulter & Wagner, 1987). The orientation of the insert in the resulting plasmid pXT-ASB was checked by digestion with the restriction enzymes *SstI* and *XhoI* + *SalI*; plasmid pXT-ASB was amplified in *Escherichia coli* strain HB101 in the presence of ampicillin (200 µg/ml). Replication-defective infectious retrovirus particles were generated by transfection of plasmid pXT-ASB into the helper-virus free-packaging cell line PA317 (Miller & Buttimore, 1986) using the calcium phosphate technique (Wigler *et al.*, 1977). At 72 h after transfection, G418 (geneticin; Gibco; 0.4 mg/ml) was added to the culture medium. Resistant cells were treated with trypsin, subcloned and recultured to approx. 50% confluency. Selection was withdrawn for 2 days and the culture medium was filtered through a 0.45 µm-pore-size filter.

Retroviral gene transfer

At 48 h before infection, human MPS VI fibroblasts were seeded at 30% confluency into a 75 mm² culture flask. Cells were incubated in 2 ml of the filtered culture supernatant of XT-ASB-producing PA317 cells for 16 h in the presence of Polybrene (10 µg/ml). At 48 h after infection, culture medium was supplemented with G418 (0.2 mg/ml), and fibroblasts were selected in comparison with an uninfected control. G418-resistant MPS VI fibroblasts were assayed for ASB activity, and expressing cells were used for further investigations.

Enzyme assays

In cell extracts, ASB activity was measured after immunoprecipitation of ASB with an ASB-specific antiserum (Steckel *et al.*, 1983) using *p*-nitrocatechol sulphate as substrate. A unit of activity is the amount of enzyme catalysing the formation of 1 µmol of *p*-nitrocatechol/min. Protein was determined by Bio-Rad protein assay (500-0006), with BSA as standard. β -Hexosaminidase activity was determined as described by von Figura (1977).

DNA preparation and Southern-blot analysis

Genomic DNA was isolated from human fibroblast lines essentially as described by Sambrook *et al.* (1989), digested with restriction endonucleases, separated by electrophoresis through a 0.6%-agarose gel and transferred to Hybond-N membranes (Amersham Corp.; Southern, 1975). Filters were hybridized for 16 h at 42 °C in 48% (v/v) formamide/10% (w/v) dextran sulphate (Pharmacia-LKB Biotechnology/4.8 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate)/1 × Denhardt's (Sambrook *et al.*, 1989)/10 mM-Tris/HCl (pH 7.4)/salmon sperm DNA (100 µg/ml)/0.1% SDS, containing (1–2) × 10⁶ c.p.m. of [³²P]DNA/ml. Filters were washed with 2 × SSC/0.1% SDS for 2 × 5 min at room temperature and with 0.2 × SSC at 65 °C for 3 × 20 min.

RNA isolation and Northern-blot hybridization

Total RNA or polyadenylated [poly(A)⁺] RNA of human fibroblasts was prepared as described by Chirgwin *et al.* (1979) and Gonda *et al.* (1982). RNA in 20 µl of 20 mM-Mops (pH 6.8)/6% formaldehyde/33% (v/v) formamide was heated for 5 min at 70 °C and electrophoresed through a 1.3% (w/v)-agarose gel containing 2.1% formaldehyde in 20 mM-Mops, pH 6.8. RNA was transferred to Hybond-N membranes, hybridized, and washed under the conditions described for Southern blots.

Metabolic labelling, preparation of cell extracts and subcellular fractionation

Cultured human fibroblasts in either 35- or 60-mm-diameter dishes were labelled with 1.8 and 4.7 MBq of [³⁵S]methionine respectively, as described by von Figura *et al.* (1983) and Lemansky *et al.* (1985). For the chase, the medium was supplemented with 1% of a 20 mg/ml solution of methionine. The cells were harvested by scraping, and cell extracts were prepared as described by Lemansky *et al.* (1985). Postnuclear supernatants obtained from metabolically labelled cells (on confluent 60 mm-diameter dishes) were fractionated by Percoll-density-gradient centrifugation as described by Waheed *et al.* (1988).

Immunoprecipitation and electrophoresis

The extract of cells, the medium and Percoll-density-gradient fractions were immunoprecipitated using an anti-ASB antiserum (Steckel *et al.*, 1983) and analysed by SDS/PAGE as described by Waheed *et al.* (1988).

Indirect immunofluorescence

Cells were grown on coverslips, fixed and permeabilized as described by Waheed *et al.* (1988). After permeabilization, cells were incubated for 1.5 h with an anti-ASB antiserum (Steckel *et al.*, 1983), followed by an incubation with tetramethyl-rhodamine-conjugated anti-rabbit IgG (Cappel) for 1 h.

RESULTS

A retroviral vector, XT-ASB (Fig. 1), was constructed for transfer of the ASB cDNA into human MPS VI fibroblasts. A DNA fragment encoding the first 29 amino acids of the signal

Fig. 1. Structure of the recombinant retrovirus XT-ASB

XT-ASB was constructed by insertion of a human ASB cDNA [preceded by a DNA fragment encoding the first 29 amino acids of the signal peptide of lysosomal acid phosphatase (Peters *et al.*, 1990)] into the retroviral expression vector pXT1 (Boulter & Wagner, 1987). The details of construction are given in the Materials and methods section. The ASB gene is expressed from the internal herpes-simplex TK promoter (TK; 3.0 kb transcript) and *neo^R* from the Moloney-murine-leukaemia-virus 5'LTR. Owing to a cryptic splice site (3') located next to *neo^R*, apart from the total genomic transcript (6.3 kb) containing the packaging signal ψ , a spliced transcript (5.8 kb) is initiated at the 5'LTR.

Table 1. ASB activity in MPS VI fibroblasts before and after retroviral-gene transfer

ASB activities are expressed as nmol of substrate cleaved/min per mg of protein. The control cells are normal fibroblasts from diagnostic sources.

Cell line	Gene transfer	ASB activity
Control	No	11.5
5343	No	0
5343,32	Yes	390
5343,C2	Yes	392
5343,C5	Yes	417
5343,D4	Yes	19.2

Fig. 2. Southern-blot analysis of DNA from normal fibroblasts (1), MPS VI fibroblasts (2) and MPS VI fibroblasts infected with recombinant retrovirus XT-ASB (3)

A 10 µg portion of the respective genomic DNA was digested with *EcoRI* (E) or *XbaI* (X) and probed with a human ASB cDNA. The 6.3 kb *XbaI* and 2.0 kb *EcoRI* fragments detected in retrovirus-infected MPS VI fibroblasts (3) correspond to the expected lengths of intact ASB-cDNA-containing provirus (see Fig. 1). The 6.1 kb *XbaI* fragment (3) is most likely due to a short deletion in a subpopulation of the provirus.

peptide of human lysosomal acid phosphatase (Pohlmann *et al.*, 1988) and amino acids 37–533 of human ASB, which previously has been shown to code for active ASB (Peters *et al.*, 1990), was inserted into the plasmid form of the retroviral expression vector pXT1 (Boulter & Wagner, 1987). pXT1 is an N₂-based retroviral vector (Keller *et al.*, 1985) providing an internal herpes-simplex-virus thymidine kinase (TK) promoter (Colbere-Garapin *et al.*, 1979; McKnight & Kingsbury, 1982) for expression of the exogenous DNA. Furthermore, a selectable neomycin-resistance gene (*neo^R*) conferring G418 resistance is expressed from the Moloney-murine-leukaemia-virus 5'LTR (long terminal repeat)

Fig. 3. Analysis of RNA from MPS VI fibroblasts before and after retroviral infection

A Northern blot of 3 µg of poly(A)⁺ RNA from uninfected MPS VI fibroblasts (5343; lane 1) and 1.2 µg of poly(A)⁺ RNA from retrovirus-XT-ASB-infected fibroblasts (5343,C5; lane 2) was hybridized with a human ASB cDNA. The expected transcripts from the integrated retrovirus, 6.3, 5.8 and 3.0 kb in length, were detected in infected fibroblasts (lane 2), whereas no transcripts were detected in uninfected cells after 2 days of exposure (lane 1). Endogenous ASB transcripts were visible after 14 days of exposure (results not shown).

Fig. 4. Processing of ASB in MPS VI fibroblasts after retroviral infection

Retrovirus-XT-ASB-infected MPS VI fibroblasts (5343,C5) were labelled for 2 h with [³⁵S]methionine and chased for 0 (–) or 16 h (+) in the presence (+) or absence (–) of 10 mM-NH₄Cl. ASB was immunoprecipitated from cell extracts and media and subjected to SDS/PAGE. The molecular mass (*M*) of the standards is given on the left and that of expressed ASB forms on the right.

of the vector. pXT-ASB DNA was transfected into the amphotropic packaging cell line PA317 (Miller & Buttimore, 1986) and recombinant retrovirus was obtained as described in the Materials and methods section. Infection of human MPS VI fibroblasts with the XT-ASB retrovirus and subsequent selection

Fig. 5. Distribution of ASB expressed in retrovirus-XT-ASB-infected MPS VI fibroblasts

Uninfected MPS VI fibroblasts (5343; I) and XT-ASB infected MPS VI fibroblasts (5343,C5; II) were metabolically labelled for 2 h with [^{35} S]methionine and chased for 16 h. The postnuclear supernatants were subjected to Percoll-density-gradient centrifugation. (a) ASB was immunoprecipitated from the gradient fractions and subjected to SDS/PAGE. The molecular mass (M) of the standards is given on the left. The arrow on the right marks the 47 kDa polypeptide of mature ASB. (b) The distribution of ASB and β -hexosaminidase activity was determined. Dense lysosomes migrate in fractions 1 and 2 and light membranes in fractions 5 and 6 under the conditions applied.

for G418 resistance resulted in normal or elevated levels of ASB activity (Table 1). Southern-blot analysis of genomic DNA was from MPS VI cell line 5343,C5, expressing large amounts of ASB after retroviral-gene transfer (Table 1), revealed, in addition to the pattern of DNA restriction fragments observed in the uninfected parental MPS VI fibroblast line 5343 and a normal fibroblast line, an *Eco*RI DNA fragment of 2.0 kb and an *Xba*I fragment of 6.3 kb hybridizing with the ASB cDNA probe (Fig. 2). These two fragments were expected for the correctly integrated retroviral vector XT-ASB (Fig. 1). An additional *Xba*I fragment of about 6.1 kb in 5343,C5 DNA is possibly due to a small deletion in a subpopulation of the integrated provirus (Fig. 2). Three transcripts from XT-ASB of expected size were detected by Northern-blot analysis of RNA from 5343,C5 fibroblasts. These transcripts are not present in the uninfected parental fibroblast line 5343 (Fig. 3). A genomic RNA of approx. 6.3 kb and a subgenomic transcript of 5.8 kb are presumably initiated at the 5'LTR of XT-ASB, whereas a 3.0 kb transcript is initiated at

Fig. 6. Immunofluorescence of ASB in retrovirus-infected MPS VI fibroblasts

MPS VI fibroblasts (a) and retrovirus XT-ASB infected MPS VI fibroblasts (b) were incubated with rabbit anti ASB antiserum, followed by tetramethylrhodamine-conjugated anti-rabbit IgG.

the internal TK promoter. On longer exposure, the endogenous transcripts of the ASB gene, 4.8, 2.5 and 1.8 kb in size (see Fig. 3 of Peters *et al.*, 1990), are visible in the RNA of uninfected 5343 fibroblasts (results not shown).

To investigate the synthesis and processing of ASB expressed in MPS VI fibroblasts after retroviral-gene transfer, 5343,C5 fibroblasts were metabolically labelled for 2 h with [^{35} S]methionine and chased for up to 16 h. After a 2 h pulse a 64 kDa polypeptide, considered to represent the ASB precursor, was immunoprecipitated from the cell extract (Fig. 4, lane 1). A polypeptide of the same size was detectable in the secretions when the cells were labelled and chased in the presence of 10 mM-NH₄Cl (Fig. 4, lane 6). NH₄Cl is known to induce secretion of precursors of lysosomal enzymes (Hasilik & Neufeld, 1980; Myerowitz & Neufeld, 1981) including those of ASB (Steckel *et al.*, 1983; Peters *et al.*, 1990; Taylor *et al.*, 1990). During a 16 h chase the 64 kDa precursor disappeared and a 47 kDa and a 12.5 kDa polypeptide accumulated in the cell extract (Fig. 4, lane 2). Localization of ASB in MPS VI fibroblasts after retroviral-gene transfer was demonstrated in two experiments. Subcellular fractionation of 5343,C5 fibroblasts by Percoll-density-gradient centrifugation revealed that approx. 60% of ASB activity was associated with the dense lysosomes, paralleling the distribution of β -hexosaminidase, a lysosomal marker (Fig. 5b, II). A similar distribution has previously been shown for ASB expressed in BHK-21 cells (Peters *et al.*, 1990). Immunoprecipitation from the gradient fractions showed that the mature 47 kDa ASB form predominantly fractionated with the dense lysosomes (Fig. 5a, II, lane 1). A small part of the 47 kDa polypeptide fractionates with the light membranes (Fig. 5a, II, lanes 5 and 6).

Furthermore, ASB was detected by indirect immunofluorescence in 5343,C5 fibroblasts, exhibiting granular distribution predominantly in the perinuclear area (Fig. 6b), typical for lysosomal distribution (Parenti *et al.*, 1987). The intensities of ASB-specific signals varied between individual cells, suggesting large differences in expression of ASB activity (results not shown). No ASB-cross-reacting material was detected in the uninfected MPS VI cell line 5343 (Fig. 6a).

DISCUSSION

The results described here demonstrate that a human ASB cDNA inserted into a retroviral vector can be efficiently transferred *in vitro* into human fibroblasts of a patient suffering from the Maroteaux-Lamy syndrome. After infection with the recombinant retrovirus, ASB enzyme activity rose from undetectable levels before gene transfer to up to 36-fold overexpression, as compared with normal fibroblasts. The retrovirus-encoded ASB polypeptides were shown to be synthesized and processed as endogenous ASB in normal fibroblasts (Taylor *et al.*, 1990) and human ASB expressed in baby-hamster-kidney cells (BHK-21; Peters *et al.*, 1990); a 64 kDa precursor was processed to the mature form of the enzyme, which is composed of two polypeptides of 47 kDa and 12.5 kDa in size. Appearance of the mature form of ASB suggests that the ASB precursor is transported to the lysosomal compartment, where proteolytic processing is accomplished. Localization of retrovirus-encoded ASB in dense lysosomes was furthermore demonstrated by Percoll-density-gradient centrifugation and indirect immunofluorescence.

Correction of enzyme deficiency *in vitro* by retroviral-gene transfer has recently been reported for two other lysosomal storage diseases. Human glucocerebrosidase has been expressed in fibroblasts (Choudary *et al.*, 1986; Sorge *et al.*, 1987) and haematopoietic cells (Fink *et al.*, 1990) from Gaucher patients. A rat β -glucuronidase cDNA has been introduced into human fibroblasts of patients with mucopolysaccharidosis type VII, as well as into fibroblasts, retinal epithelia cells and haematopoietic cells of a β -glucuronidase-deficient dog (Wolfe *et al.*, 1990). Histochemical staining revealed that only a subpopulation of the treated cells contained β -glucuronidase activity, paralleling our observation of large differences in ASB-specific staining between individual cells in indirect immunofluorescence.

The retroviral vector construct XT-ASB described here may be used for transfer of the ASB cDNA into haematopoietic cells of the feline MPS VI model. Subsequent autologous bone-marrow transplantation could help to evaluate the prospects of somatic gene therapy of Maroteaux-Lamy disease in humans. However, efficient infection of uncommitted haematopoietic stem cells and maintenance of high levels of expression of the transferred gene over long periods of time represent major obstacles recently encountered in gene-transfer experiments in dogs and monkeys (Kantoff *et al.*, 1987; Stead *et al.*, 1988).

Skin fibroblasts may be an attractive alternative tissue for gene-therapy approaches to lysosomal storage diseases (Osborne & Miller, 1988; Friedmann, 1989). In the feline MPS VI model, fibroblasts reimplanted into the animal after correction of the ASB-deficiency *in vitro* could serve as a source of the enzyme. To increase the amount of enzyme secreted into the circulation, the gene for the M_r -46000 mannose 6-phosphate receptor could be transferred into the fibroblasts along with the ASB cDNA (Chao *et al.*, 1990) and thereby extend the supply of ASB available for endocytosis into affected cells.

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