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Research Article

Mucopolysacchariodosis type VI (MPS VI) is the lysosomal storage disorder caused by the deficient activity of arylsulfatase B (ASB; N-acetylgalactosamine 4-sulfatase) and the subsequent accumulation of the glycosaminoglycan (GAG), dermatan sulfate. In this study, a retroviral vector containing the full-length human ASB cDNA was constructed and used to transduce skin fibroblasts, chondrocytes, and bone marrow cells from human patients, cats, or rats with MPS VI. The ASB vector expressed high levels of enzymatic activity in each of the cell types tested and, in the case of cat and rat cells, enzymatic expression led to complete normalization of 35SO4 incorporation. In contrast, overexpression of ASB in human MPS VI skin fibroblasts did not lead to metabolic correction. High-level ASB expression was detected for up to eight weeks in transduced MPS VI cat and rat bone marrow cultures, and PCR analysis demonstrated retroviral-mediated gene transfer to approximately 30-50% of the CFU GM-derived colonies. Notably, overexpression of ASB in bone marrow cells led to release of the enzyme into the media and uptake by MPS VI cat and rat skin fibroblasts and/or chondrocytes via the mannose-6-phosphate receptor system, leading to metabolic correction. Thus, these studies provide important rationale for the development of gene therapy for this disorder and lay the frame-work for future in vivo studies in the animal model systems.

Arylsulfatase B Activities and Glycosaminoglycan Levels in Retrovirally Transduced Mucopolysaccharidosis Type VI Cells

Prospects for Gene Therapy

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Abstract

Mucopolysacchariodosis type VI (MPS VI) is the lysosomal storage disorder caused by the deficient activity of arylsulfatase B (ASB; *N*-acetylgalactosamine 4-sulfatase) and the subsequent accumulation of the glycosaminoglycan (GAG), dermatan sulfate. In this study, a retroviral vector containing the full-length human ASB cDNA was constructed and used to transduce skin fibroblasts, chondrocytes, and bone marrow cells from human patients, cats, or rats with MPS VI. The ASB vector expressed high levels of enzymatic activity in each of the cell types tested and, in the case of cat and rat cells, enzymatic expression led to complete normalization of $^{35}SO_4$ incorporation. In contrast, overexpression of ASB in human MPS VI skin fibroblasts did not lead to metabolic correction. High-level ASB expression was detected for up to eight weeks in transduced MPS VI cat and rat bone marrow cultures, and PCR analysis demonstrated retroviral-mediated gene transfer to ~ 30–50% of the CFU GM-derived colonies. Notably, overexpression of ASB in bone marrow cells led to release of the enzyme into the media and uptake by MPS VI cat and rat skin fibroblasts and/or chondrocytes via the mannose-6-phosphate receptor system, leading to metabolic correction. Thus, these studies provide important rationale for the development of gene therapy for this disorder and lay the frame-work for future in vivo studies in the animal model systems. (*J. Clin. Invest.* 1996. 98:497–502.) Key words: lysosomal storage diseases • animal models • gene therapy

Introduction

Mucopolysaccharidosis type VI (MPS VI; Maroteaux-Lamy disease)[1] is the lysosomal storage disease resulting from the deficient activity of arylsulfatase B (ASB; *N*-acetylgalactosamine-4-sulfatase; E.C. 3.1.6.1) (1). ASB hydrolyzes sulfate esters from glycosaminoglycans (GAGs), principally dermatan sulfate. Thus, an inherited deficiency of ASB activity leads to the accumulation of dermatan sulfate in various cell types. In man, MPS VI is characterized by short stature, dysostosis multiplex, coarse facial features, cardiac valve anomalies, thickening of the tracheal wall and corneal clouding (1). Notably, MPS VI has been described in cats, rats and dogs, and breeding colonies of the cats and rats have been established (2–4).

Several approaches have been considered and/or evaluated for the treatment of MPS VI, including bone marrow transplantation (BMT), enzyme replacement and hematopoietic stem cell-mediated gene therapy. BMT has been attempted in human patients, cats and rats as a means of providing a source of normal cells that could release ASB for correction of the target sites of pathology, such as the articular cartilage, heart valves, and cornea. Follow-up of one human patient who was transplanted at 12 years of age, and expressed normal levels of ASB in leukocytes for up to 40 mo after transplantation, documented significant improvement of cardiac and respiratory functions, reduced hepatosplenomegaly, and some improvement in joint mobility. However, no changes in the bone pathology, facial appearance, corneal clouding, or short stature were observed (5). Analysis of several transplanted MPS VI rats also has indicated that BMT does not lead to widespread improvement of the skeletal lesions (unpublished data).

The full-length cDNA and/or genomic sequences encoding human, cat and rat ASB have been isolated and expressed (6–11), and several mutations causing the human and animal disorders have been described (e.g., 12, 13). Moreover, ASB retroviral vectors have been constructed and used to transduce skin fibroblasts from MPS VI patients (14, 15). A surprising outcome of these latter experiments was that overexpression of ASB in human MPS VI skin fibroblasts did not reduce the level of $^{35}SO_4$ incorporation (15).

The goals of the current study were to: (*a*) construct an ASB retroviral vector that could be used for overexpression of the human enzyme in various cell types; (*b*) compare the transduction efficiencies and expression levels in human, cat and rat MPS VI skin fibroblasts, chondrocytes and bone marrow cells and evaluate whether there were species-specific and/or cell type-specific differences in the levels of activity obtained and effects on $^{35}SO_4$ incorporation; and (*c*) determine whether transduced MPS VI bone marrow cells could release ASB and, if so, whether the released enzyme could be taken up by MPS VI skin fibroblasts and/or chondrocytes, two primary cell targets for gene therapy. The results described in this manuscript lay the foundation for future in vivo studies in the animal model systems and provide important rationale for the development of gene therapy in this disorder.

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1. *Abbreviations used in this paper:* ASB, arylsulfatase B; BMT, bone marrow transplantation; GAG, glycosaminoglycan; LTMCM, long term marrow culture media; MPS VI, mucopolysaccharidosis type VI.

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Methods

Construction of the ASB/MFG retroviral vector. To insert the full-length human ASB cDNA (6) into the MFG vector (kindly supplied by Dr. Paul Robbins, University of Pittsburgh), the wild-type ASB sequence was modified to introduce an NcoI restriction site into the translation initiation codon. An ASB cDNA fragment containing the modified sequence was generated by PCR mutagenesis (16) using sense (5′-CTCGAGACCATGGGTCCGCGCGGCGCG-3′) and antisense (5′-GCTTTTTAAAGCTGCAGTGAC-3′) primers containing flanking XhoI and PstI restriction sites, respectively (underlined). The mutagenized fragment was then ligated to a partial ASB cDNA to construct a full-length sequence. After confirming the presence of the NcoI site by dideoxy DNA sequencing (17), the modified, full-length human cDNA was ligated to NcoI/BamHI-digested MFG vector.

To generate packaging cell lines producing the ASB/MFG recombinant virus, the ASB/MFG vector was co-electroporated with the pSV2Neo (Pharmacia, Piscataway, NJ) plasmid (molar ratio 20:1, respectively) into the envAm12 amphotropic packaging cell line (18), kindly supplied by Dr. Arthur Bank (Columbia University). After G418 selection (400 μg/μl), 14 resistant colonies were isolated and the ASB activities were determined using *p*-nitrocatechol sulfate (19). Virus-containing media was collected from each of these packaging cells and used to transduce NIH 3T3 cells; the packaging cell line resulting in the highest level of ASB expression (i.e., AMFG-1) was used for subsequent experiments. Protein determinations were performed using a Bio-Rad protein assay kit according to the manufacturer's instructions.

Retroviral transduction of skin fibroblasts. Retroviral transduction of human, cat and rat MPS VI skin fibroblasts was carried out using standard methods (e.g., 20). Briefly, human and rat fibroblasts were grown in DMEM culture media (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cat fibroblasts were grown in RPMI media (GIBCO BRL) containing 15% FBS, 5% fetal cat serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. To transduce these cultures with the ASB/MFG retroviral vector, cells were grown to ~ 30% confluency and viral producer cell media containing 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO) was added. The cells were grown in the presence of fresh virus-containing media for ~ 24 h, at which time the viral producer media was replaced with normal culture media. Transduced cells were then grown until they reached mid-confluency, harvested by trypsinization and analyzed for ASB expression and/or $^{35}SO_4$ incorporation (see below).

Establishment of cat and rat bone marrow cultures. Cat bone marrow was obtained under general anesthesia from proximal humeri and femurs. Light-density mononuclear cells were isolated by centrifugation over Ficoll (density 1.077 grams/ml), washed twice with Hank's solution and counted by trypan blue exclusion (21). The cells were plated at a density of $\sim 2 \times 10^6$ per ml in Iscove's modified Dulbecco's medium (GIBCO BRL) containing 12.5% each of heat inactivated FBS and horse serum, 40 μg/ml myoinositol, 1 mg/ml folic acid, 400 μg/ml glutamine, 1% penicillin/streptomycin/fungizone (GIBCO BRL), 0.45 mM sodium chloride, 10^{-3} mM hydrocortisone, 0.1 mM β-mercaptoethanol, and 2 ng/ml canine stem cell factor (a gift from Amgen Inc.). This was designated long term marrow culture media (LTMCM). Feline bone marrow cells were grown in LTMCM at 37°C in the presence of 5% CO_2.

Rat bone marrow was obtained from the femurs of sacrificed animals. Ficoll gradient isolation of mononuclear cells and the cell growth conditions were essentially the same as that described for cats, except that the LTMCM contained 12.5 ng/ml (250 U) of murine interleukin 3 (GIBCO BRL), 2.5 ng/ml (250 U) of murine interleukin 6 (Genzyme Corp., Boston, MA), and 5 ng/ml (500 U) of murine stem cell factor (Stem Cell Technologies). CFU GM-derived colonies were grown in methylcellulose-containing media as previously described (22).

Retroviral transduction of bone marrow cells. The method used to transduce cat bone marrow cells was essentially the same as that described by Bienzle et al. (23). One day before the infection, LTMCM was added to the ASB/MFG producer cells. Bone marrow cultures were initiated the next day by plating $\sim 1 \times 10^8$ mononuclear cells in 60 ml of this fresh virus-containing media (a final concentration of $\sim 2 \times 10^6$ target cells/ml of virus-containing media) in the presence of 4 μg/ml polybrene. On day 3, 30 ml of the media was removed and the nonadherent cells were harvested and resuspended in 30 ml of fresh virus-containing media, which was then added back to the bone marrow cultures. Stromal layers generally became visible in these cultures by day 4. On day 6, the cultures were demidepopulated by removing 30 ml of media containing the nonadherent cells and replacing it with fresh media. 24 h later, 30 ml of the media was replaced with fresh virus-containing media. This process (i.e., demidepopulation and retransduction) was repeated on days 11/12. Thus, each culture of cat bone marrow cells was transduced four times with fresh virus-containing media (days 1, 3, 7, and 12). Retroviral transduction of the rat bone marrow cells was performed essentially as described above, except that the initial cultures were seeded in 75 mm^2 culture flasks containing 10 ml of virus-containing media (a final concentration of $\sim 1 \times 10^6$ target cells/ml of virus-containing media).

PCR analysis of CFU GM-derived colonies. CFU GM-derived colonies were grown from the transduced cat and rat bone marrow cultures. Individual CFU GM-derived colonies were isolated, washed with PBS, and microcentrifuged for 1 min at 10,000 *g*. The pellets were resuspended in 20 μl of PCR buffer (10 mM Tris, pH 8.3, containing 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween 20, and 50 μg/ml of proteinase K), and cell lysates were prepared by incubation at 55°C for 2 h. The proteinase K was then inactivated (95°C for 10 min), and PCR amplification (24) was carried out for 50 cycles (each cycle consisted of denaturation at 98°C for 10 min and 94°C for 1 min, annealing at 68°C for 1 min and extension at 72°C for 2 min). Sense and antisense PCR primers (5′-GGTCGGAAAATGGCACCTGGGAA-3′ and 5′-CCCTCGGACGCCTCCTTCCCACA-3′) were constructed from exons 2 and 6, respectively. Thus, only the retrovirally-encoded ASB cDNA could be amplified by this reaction since the two primers were separated by several large introns (> 30 kb) in the genomic sequence.

Rat chondrocyte cultures and retroviral transduction. Chondrocyte cultures were established from normal and MPS VI rats according to the method of Tsukazaki et al. (25), with several minor modifications. Articular cartilage was dissected from the knee joints of ~ 10 week old rats and digested for 30 min at 37°C in 0.25% trypsin and 0.01 M EDTA. The digested material was collected by centrifugation (450 *g* for 15 min), resuspended in 0.2% collagenase prepared in 1:1 Ham's F-12 (GIBCO BRL)/DMEM media, and incubated for an additional 4 h at 37°C. The mixtures were centrifuged slowly (450 *g*) to remove debris, and the supernatants were removed and recentrifuged at 1000 *g* to harvest the remaining cells. The cell pellets were washed twice with serum free media and plated at a density of $\sim 1 \times 10^6$ cells/ml in media containing antibiotics (1% penicillin/streptomycin) and 10% fetal calf serum. Generally, each culture was established from a pool of six normal or MPS VI rats and could be grown for up to 3 wk. Retroviral transductions were performed as described above for primary skin fibroblasts.

$^{35}SO_4$ incorporation. To evaluate the turnover of GAGs in the various cell lines, cells were grown to ~ 30% confluency in their normal culture media, which was then replaced with Ham's F-12 (sulfate free) media containing 10% fetal bovine serum and 100 U/ml penicillin G (GIBCO BRL). $Na_2{}^{35}SO_4$ (20 μCi/ml; Dupont/New England Nuclear, Boston, MA) was added to the media and incubated with the cells for 72 h. After labeling, this media was replaced with standard culture media and the cells were grown for an additional 4 d. Cell extracts were prepared by freeze-thaw fracture and debris was removed by centrifugation (1,000 *g*). Radioactivity was then determined in the supernatants using a model 1219 Rackbeta scintillation counter (Pharmacia).

Table I. ASB Activities and $^{35}SO_4$ Incorporation in Normal, MPS VI and Retrovirally Transduced MPS VI Fibroblasts

Cell line	ASB activities	$^{35}SO_4$ incorporation
	nmol/min/mg protein	*cpm/μg protein*
Human		
Normal	42 (36–45	4 (2–6)
MPS VI	6 (3.5–10)	97 (79–105)
Transduced MPS VI	430 (410–565)	94 (83–112)
Cat		
Normal	24 (19–27)	5 (3.5–7)
MPS VI	4 (3–7.5)	125 (88–146)
Transduced MPS VI	400 (344–468)	15 (9–21)
Rat		
Normal	38 (30–41)	7 (2–11)
MPS VI	6 (5.5–7)	130 (95–167)
Transduced MPS VI	350 (290–403)	6 (3–9)

ASB activities and $^{35}SO_4$ incorporation were determined as described in the Methods. The average values were derived from three experiments. Ranges are shown in parentheses.

Cross-correction studies. For the cross-correction studies, MPS VI cat and rat bone marrow cells were retrovirally-transduced as described above. To harvest the released ASB for cross-correction, the media was changed to serum-free/sulfate-free Ham's F-12/DMEM (1:1) and grown for 24 h at 37°C. The ASB-containing media was then added to cultures of non-transduced MPS VI cat or rat fibroblasts and/or chondrocytes, which were grown for an additional 72 h in the presence of $Na_2{}^{35}SO_4$ (20 μCi/ml) with or without 5 mM mannose 6-phosphate (Sigma Chemical Co.). The media was then changed and the cells were grown for another 72 h before the ASB activities and $^{35}SO_4$ incorporation levels were determined.

Results

Retroviral transduction of MPS VI skin fibroblasts. The full-length human ASB cDNA was inserted into the MFG vector and several amphotropic producer cell lines were isolated. Transduction of NIH 3T3 cells with media from producer line AMFG-1 resulted in high-level ASB expression (not shown), and this producer line was therefore used as the source of recombinant virus for the remainder of the studies. Table I summarizes the ASB activities and $^{35}SO_4$ incorporation levels in human, cat and rat MPS VI skin fibroblasts after transduction with the AMFG-1 virus. Note that high-level ASB activity was obtained in each of the transduced cell lines (> 15-fold above normal) and, in the case of cat and rat fibroblasts, overexpression of ASB led to correction of the $^{35}SO_4$ incorporation (a measure of GAG accumulation). In contrast, retrovirally transduced human MPS VI cells overexpressing ASB did not show evidence of corrected $^{35}SO_4$ incorporation. These results were not due to increased release of other GAG hydrolyzing enzymes into the media, but may relate to the fact that there was a partial reduction of the intracellular activity of arylsulfatase A, another lysosomal sulfatase, in cells overexpressing ASB (not shown). This is consistent with previously reported results which demonstrated that overexpression of human ASB in human skin fibroblasts led to a reduction of other sulfatase activities and no correction of $^{35}SO_4$ incorporation (15).

Retroviral transduction of MPS VI chondrocytes. Chondrocyte cultures were established from normal and MPS VI rats and the level of ASB activity and $^{35}SO_4$ incorporation were determined (Fig. 1). Notably, the levels of $^{35}SO_4$ incorporation in normal rat chondrocytes was ~ 20-fold greater than normal fibroblasts (see Table I). Moreover, increased levels of $^{35}SO_4$ levels in the MPS VI chondrocytes was ~ 40-fold greater than that observed in MPS VI fibroblasts. This is consistent with the fact that chondrocytes synthesize and secrete large amounts of GAGs and are a primary site of pathology in the mucopolysaccharidoses (1). Retroviral transduction of rat MPS VI chondrocytes with the human ASB/MFG vector led to high-level ASB activity and complete correction of the $^{35}SO_4$ incorporation (Fig. 1).

Retroviral transduction of MPS VI bone marrow cells. To evaluate whether the ASB/MFG vector could express high levels of human ASB activity in MPS VI hematopoietic cells, bone marrow cultures were established from MPS VI cats and rats. To optimize gene transfer to progenitor and/or stem cells in these cultures, a four "cycle" infection procedure was used (see Methods for details). Note that greater than normal levels of ASB activity could be obtained by the third transduction (Table II). Repeated transductions did not lead to increased ASB activity (not shown).

To evaluate the stability of human ASB expression in the retrovirally-transduced cat and rat MPS VI bone marrow cultures, the transduced cells were grown for up to eight weeks in LTMCM and monitored for ASB activity (Table II) and the presence of the ASB/MFG retrovirus by PCR (not shown). Notably, transduced cells were detected throughout the eight week growth period and high-level ASB expression was maintained.

To evaluate these findings further, PCR analysis also was performed on individual CFU GM-derived colonies grown from the retrovirally-transduced cells (Table III). Note that PCR-positive CFU GM-derived colonies were detected throughout the eight-week period and that a 30–50% gene transfer efficiency was achieved. This data demonstrate significant gene transfer to committed progenitor cells using the four cycle infection procedure.

Cross-correction studies. To evaluate whether ASB was released by retrovirally-transduced MPS VI bone marrow cells and, if so, whether the released enzyme could be taken up by non-transduced MPS VI skin fibroblasts and/or chondrocytes for metabolic correction, a series of "cross-correction" experiments were undertaken (Fig. 2). For these studies, transduced bone marrow cells were grown in serum-free/sulfate-free media for 24 h. The ASB-containing media was then transferred to nontransduced cat (fibroblasts) or rat (chondrocytes) MPS VI cells, which were grown in the presence of $^{35}SO_4$ with or without mannose-6-phosphate. Note that the "cross-corrected" MPS VI cells had normal levels of ASB activity, and that the uptake of the released enzyme could be inhibited by mannose-6-phosphate (Fig. 2 *A*). Consistent with the high-level ASB expression, $^{35}SO_4$ incorporation was normalized in the "cross-corrected" cat and rat cells (Fig. 2 *B*).

Discussion

MPS VI is a disorder with multiple sites of pathology, including the bones, joints, skin, heart, liver, spleen and cornea. To effectively treat this disorder, ASB activity must be expressed at these various sites during the appropriate time in develop-

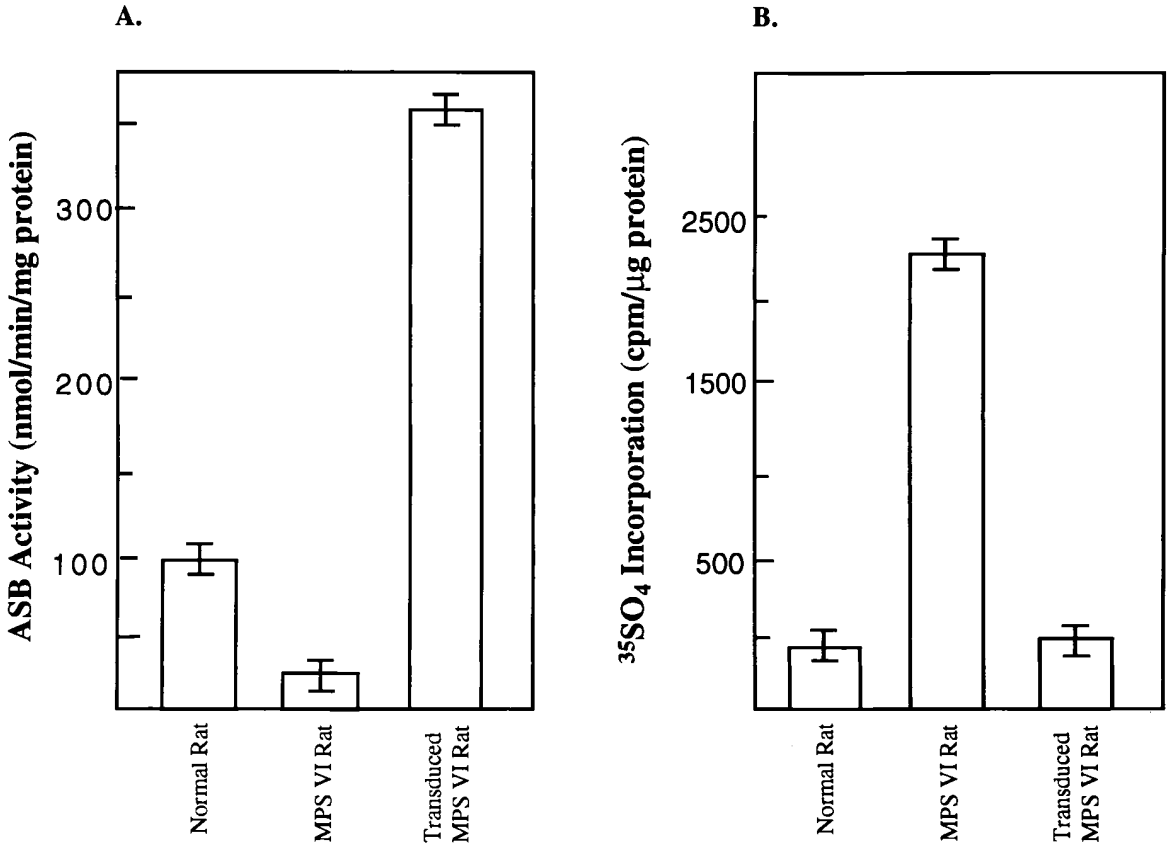

Figure 1. Retroviral transduction of MPS VI chondrocytes. Chondrocyte cultures were established from normal and MPS VI rats as described in the Methods and transduced with the ASB/MFG vector. Each culture was derived from a pool of six rats. ASB activities (*A*) and $^{35}SO_4$ incorporation (*B*) were determined as described in the Methods. Each transduction experiment was repeated three times and the bars indicate the mean values±one standard error.

ment. At present, autologous transplantation of genetically corrected hematopoietic stem cells remains one of the few approaches to accomplishing such wide-spread gene delivery.

The results of BMT in animal models of lysosomal storage diseases demonstrates the feasibility of this approach and provides rationale for the development of hematopoietic stem cell gene therapy (for review see reference 26). Guiding principles which have emerged from these transplantation studies include the facts that: (*a*) newborn transplants are more effective than those performed on adults since reversal of pathology is much more difficult to achieve than prevention; this is particularly true for the treatment of bone and dense connective tis-

Table II. ASB Activities in Normal, MPS VI, and Retrovirally Transduced MPS VI Cat and Rat Bone Marrow Cultures

	ASB Activities (nmol/min/mg protein)					
	MPS VI		Normal		Transduced MPS VI	
	Cats	Rats	Cats	Rats	Cats	Rats
Infection						
1	17 (14–19)	4.5 (3.1–5.2)	152 (113,191)	421 (333–496	135 (113–162)	92 (83–123)
2	25 (21–28)	4.0 (2.9–4.9)	145 (103,187	398 (310–446)	265 (241–295)	298 (215–334)
3	36 (30–40)	4.5 (2.9–5.4)	185 (155,215)	445 (403–501)	432 (398–468)	530 (516–608)
4	38 (31–42)	5.0 (4.1–6.0)	112 (94,130)	408 (365–430)	537 (379–674)	488 (415–524)
wk 8	33 (26–36)	5.0 (4.0–5.7)	127 (107,147)	434 (400–496)	553 (469–637)	446 (430–495)

The cat and rat bone marrow cultures were grown and transduced as described in the Methods. For the MPS VI and normal cells, mock transductions were carried out using the MFG vector alone. For the transduced MPS VI cells, the ASB/MFG vector was used. The values for wk 8 represent the ASB activities determined eight weeks (56 d) after the first transduction. All values were derived from the non-adherent cells, except for wk 8, when the adherent cells were harvested and assayed. Average values are shown, and the ranges are indicated in the parentheses.

Table III. PCR Analysis of CFU GM-Derived Colonies Grown From Retrovirally Transduced Cat and Rat MPS VI Bone Marrow Cultures

Animal model	Days in culture				
	I_1	I_2	I_3	I_4	*wk 8*
Cat	18/43 (42%)	20/38 (53%)	7/18 (39%)	8/17 (47%)	nd
Rat	30/57 (52%)	20/46 (43%)	13/36 (28%)	14/30 (30%)	16/30 (53%)

Cat and rat bone marrow cultures were grown and transduced as described in the Methods. 3 d after each transduction (I_1–I_4), nonadherent cells were harvested and CFU GM-derived colonies were grown in methylcellulose. Individual CFU GM-derived colonies were picked and analyzed by PCR for the presence of the ASB/MFG vector as described in the Methods. wk 8 represents 56 d after the first transduction. *nd*, not determined.

sue disorders (such as the mucopolysaccharidoses), as well as those with neurologic lesions (e.g., 27, 28); and (*b*) the success of BMT is disease-specific and relates directly to the ability of the normal enzyme to be released from the transplanted cells and "cross-correct" diseased cells (e.g., 29). This may be particularly true for the mucopolysaccharidoses, since many of the target sites of pathology are nonhematopoietic.

The current studies were carried out to undertake the development of hematopoietic stem cell gene therapy for MPS VI. The ASB/MFG vector expressed high levels of human ASB activity in skin fibroblasts, chondrocytes and/or bone marrow cultures established from MPS VI patients, rats and cats. Notably, high-level enzymatic expression in MPS VI cat and rat cells led to metabolic correction, as judged by $^{35}SO_4$ incorporation, whereas overexpression of ASB in human skin fibroblasts did not.

Similiar observations had been made previously using human skin fibroblasts (15), where it was shown that overexpression of ASB in MPS VI cells led to a reduction of other sulfatase activities and a secondary accumulation of GAGs. Our data are consistent with these findings, but demonstrate their species-specific nature. While these in vitro results are intriguing, it remains to be seen how they relate to the in vivo situation.

Another finding described in this manuscript is the fact that ASB can be released from retrovirally-transduced bone marrow cells and taken up by nontransduced skin fibroblasts and chondrocytes, two important cellular targets for gene therapy. As noted above, the ability of a lysosomal enzyme to be released from transplanted hematopoietically-derived cells is critical to the success of stem cell-mediated gene therapy. Although no attempts were made to quantitate the amount of enzyme released from the transduced bone marrow cells, enough enzyme was obtained within 24 h to completely normalize the ASB activity and $^{35}SO_4$ incorporation in MPS VI cells. We also demonstrated that the released enzyme was internalized via the mannose-6-phosphate receptor, to our knowledge, the first demonstration of receptor-mediated enzyme uptake by chondrocytes. These results have important implications for enzyme replacement therapy, as well as gene therapy, since they clearly demonstrate that intravenously delivered enzyme can be taken up by these important cellular targets. However, it

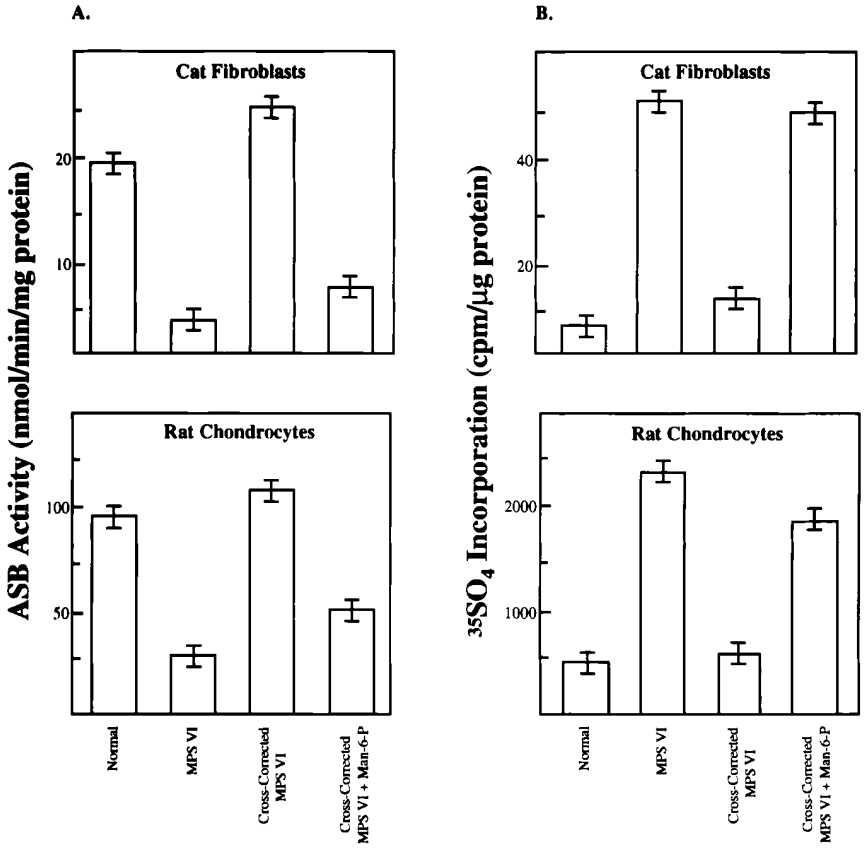

Figure 2. Cross-correction of MPS VI skin fibroblasts and chondrocytes. Retrovirally-transduced rat MPS VI bone marrow cells were grown for 24 h in sulfate free/serum free Ham's F-12 media, which was then harvested and transferred onto the nontransduced MPS VI cat (fibroblast) and rat (chondrocyte) cultures. The "cross-corrected" cells were then grown for an additional 72 h in the presence of $Na_2{}^{35}SO_4$ with or without mannose-6-phosphate, and then chased for an additional 72 h. Each uptake experiment was repeated three times and the bars indicate the mean values±one standard error.

also remains to be seen how these results relate to in vivo therapy, particularly given the fact that a large diffusion barrier exists between the cartilage and circulatory system and the released enzymes are unlikely to "travel" far in well-formed, dense connective tissue. In fact, in utero therapy, initiated either before or simultaneous with the formation of dense connective tissue, may be required in order to effectively treat the debilitating skeletal lesions in this disorder.

Thus, these studies lay the foundation for the development of hematopoietic stem cell-mediated gene therapy for MPS VI. Future studies in the animal models will identify the optimal ASB retroviral vectors for in vivo expression, define the levels of ASB activity that are therapeutic, and identify the developmental window during which therapeutic intervention must be undertaken. Fundamental information concerning the feasibility of this approach should be obtained, leading to the development of gene therapy in human patients.

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