Restoration of normal lysosomal function in mucopolysaccharidosis type VII cells by retroviral vector-mediated gene transfer

(gene therapy/lysosomal storage diseases/substrate degradation/ β -glucuronidase/animal models)

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ABSTRACT Retroviral vectors were constructed containing a rat β -glucuronidase cDNA driven by heterologous promoters. Vector-mediated gene transfer into human and canine β -glucuronidase-deficient mucopolysaccharidosis type VII fibroblasts completely corrected the deficiency in β -glucuronidase enzymatic activity. In primary cultures of canine mucopolysaccharidosis type VII retinal pigment epithelial cells, which contain large amounts of undegraded glycosaminoglycan substrates, vector correction restored normal processing of specific glycosaminoglycans in the lysosomal compartment. In canine mucopolysaccharidosis type VII bone marrow cells, β -glucuronidase was expressed at high levels in transduced cells. Thus, the vector-encoded β -glucuronidase was expressed at therapeutic levels in the appropriate organelle and corrected the metabolic defect in cells exhibiting the characteristic pathology of this lysosomal storage disorder.

The lysosomal storage disorders result from genetic deficiencies in the activities of specific lysosomal hydrolases or the defective transport of these enzymes to lysosomes (1). In diseased cells, the degradation of metabolites is blocked and specific substrates accumulate in the lysosomes (1). A promising approach to the treatment of these as well as other inborn errors of metabolism is somatic cell gene therapy (2–4). Gene transfer by retroviral vectors has shown that normal genes can be transferred to, and expressed in, cells derived from genetically diseased humans and animals (3–10). For gene transfer to be effective in the lysosomal storage disorders, the product of the transferred gene must be catalytically active toward the stored natural substrate in the lysosomes of diseased cells.

To investigate the ability of retroviral vector-mediated gene transfer to restore the enzymatic deficiency and to correct the metabolic defect associated with a lysosomal storage disease, mucopolysaccharidosis type VII (MPS VII; Sly disease) (11) was used as a model system. In MPS VII, deficiency of β -glucuronidase (GUS) activity results in the lysosomal accumulation of unmetabolized glycosaminoglycans (GAGs) (12). The MPS VII model has the following features that are useful for gene transfer studies: (i) the pathologic phenotype is retained in certain cells in culture; (ii) GUS cDNAs have been isolated (13–16); and (iii) canine (17) and murine (18) models of MPS VII have been identified.

A rat GUS cDNA (13) was transferred by retroviral vectors into human and canine GUS-deficient MPS VII cells. The retroviral vector-mediated gene transfer resulted in the expression of high levels of GUS enzymatic activity in MPS VII fibroblasts, retinal pigment epithelial (RPE) cells, and

bone marrow cells. In primary cultures of MPS VII cells containing large amounts of stored GAGs, vector correction restored normal processing of specific GAGs in the lysosomal compartment.

MATERIALS AND METHODS

Vector Construction. Vectors were constructed from N2 vectors (19) containing promoters from the simian virus 40 (SV-40) virus (NSV vector) or from the herpes simplex virus thymidine kinase gene (NTK vector) inserted into the *Xho* I site between the neomycin-resistance (neo) gene and the 3' long terminal repeat (LTR). A 2.1-kilobase *Eco*RI–*Oxa*NI fragment containing the rat GUS cDNA (13) (a gift of R. Andy, New York University) was end-filled and inserted into each vector by blunt-end ligation. The cDNA was cloned into a unique *Bgl* II site in the NTK vector. In the NSV vector a *Hind*III cloning site, made unique by deleting the *Hind*III site in the tetracycline-resistance gene of pBR322, was used. Recombinant plasmids were cloned in JM109 *Escherichia coli* and restriction analysis was used to identify vectors containing the GUS cDNA in the sense orientation with respect to the promoter.

Vector Virus Production. Recombinant plasmids with the correct structure were transfected by calcium phosphate precipitation into ecotropic ψ 2 packaging cells (20) and selected in G418 (0.8 mg/ml). Greater than 100 colonies of G418-resistant cells were pooled, the culture supernatant (0.45- μ m filtered) was used to infect amphotropic PA-317 packaging cells (21) in Polybrene (8 μ g/ml), and the cells were G418-selected. Ten colonies were isolated for each vector and grown as separate cell lines. Vector virus titers were determined by infecting NIH 3T3 cells with serial dilutions of vector virus, exposing the cells to G418, and counting colonies.

Cells. Human MPS VII fibroblasts were obtained from the Coriell Institute and normal human fibroblasts were established from skin biopsies. Canine MPS VII fibroblasts were established from a bone marrow biopsy and normal canine fibroblasts were established from a muscle biopsy. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) for murine and canine cells or 20% FBS for human cells, penicillin (100 units/ml), and streptomycin (100 μ g/ml). RPE cell cultures were established from the eyes of adult normal or

Abbreviations: MPS VII, mucopolysaccharidosis type VII; GAG, glycosaminoglycan; cfu, colony-forming unit; RPE, retinal pigment epithelial; SV-40, simian virus 40; LTR, long terminal repeat; GUS, β -glucuronidase; IDUA, α -L-iduronidase.

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MPS VII dogs (22, 23) and were maintained in medium supplemented with 20% FBS and antibiotics until confluent, when the concentration of FBS was reduced to 5%. Bone marrow cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS, 10% (vol/vol) heat-inactivated autologous dog serum, and antibiotics. The methylcellulose cultures also included 10% (vol/vol) culture supernatant from CA5637 cells as a source of growth factors for the granulocyte-macrophage lineage (24).

Vector Virus Infections. Fibroblasts and RPE cells were infected at 30% confluency with vector viruses [$>1 \times 10^6$ colony-forming units (cfu)/ml] in Polybrene (8 μ g/ml) at 37°C for 2–4 hr. The cells were incubated in fresh medium overnight, G418 (0.8 mg/ml) was added, and medium containing G418 was changed every 2–3 days until the cells reached confluency. RPE cells were harvested after trypsin treatment, split 1:3, seeded into 35-mm dishes in medium containing G418, and grown to confluency for labeling studies.

Bone marrow cells were infected by incubating them in filtered virus-containing supernatant (10 cfu of vector virus per nucleated cell) and Polybrene (8 μ g/ml) at 37°C on a rotating platform to keep the cells in suspension. Cells were infected for 4 hr and centrifuged, the supernatant was discarded, and cells were infected with a second batch of vector-virus-containing medium for 12 hr. Cells were pelleted by centrifugation, resuspended in growth medium, seeded into flasks for liquid cultures or into 1% methylcellulose cultures, and cultured for 12–13 days.

Enzyme Activity Determinations. Activities of GUS and α -L-iduronidase (IDUA) were measured using the 4-methylumbelliferyl β -D-glucuronide and 4-methylumbelliferyl α -L-iduronide, respectively (25, 26). Protein concentrations were measured by a modified fluorescamine assay (27). To identify GUS activity in individual cells, naphthol AS-BI β -D-glucuronide (Sigma) was used in a post-coupling reaction (28, 29). The only modification was for bone marrow cells, which were harvested by centrifugation, washed, and resuspended in isotonic phosphate-buffered saline (PBS), allowed to settle onto glass slides, and air-dried before staining. For the methylcellulose cultures of bone marrow cells, the methylcellulose containing the colonies of cells was removed by aspiration and diluted 1:15 in PBS to separate the cells from the matrix. By using this procedure, fibroblasts remained attached to the surface of the plate.

GAG Analysis. Metabolic labeling of confluent cultures of RPE cells with $^{35}\text{SO}_4$ (40 μ Ci/ml; New England Nuclear; 720 mCi/mmol; 1 Ci = 37 GBq) was performed in reduced-sulfate medium for 72 hr and the sulfated GAGs contained in the medium and cell layer of each culture dish were analyzed separately (22, 30). Enzymes were inactivated by boiling and the sulfated GAGs were precipitated using the cetylpyridinium chloride method (22). GAGs were electrophoresed on cellulose acetate and total GAGs were visualized by Alcian blue staining. Radioactive GAG bands, visualized by fluorography, were cut from the cellulose acetate sheet and quantitated by liquid scintillation spectroscopy (22). Total proteins were measured in a Bio-Rad assay.

RESULTS

Retroviral vectors for transferring the GUS cDNA were constructed from the N2 vector (19). The coding sequence from a rat GUS cDNA (13) was cloned into the NTK vector and the NSV vector to yield the vector constructs NTK-BGEO and NSV-BGEO, respectively (Fig. 1). The vectors were converted to corresponding viruses as described (21). Ten colonies of G418-resistant PA-317 cells were isolated for each vector and grown as individual cell lines. Each cell line was assayed for virus production by G418 resistance in NIH

FIG. 1. Construction and production of retroviral vectors containing a rat GUS cDNA. By using the N2 vector (19) as a backbone, the strong transcriptional promoters from the SV-40 virus (NSV vector) and from the herpes simplex virus thymidine kinase gene (NTK vector) were inserted in the *Xho* I site between the neo gene and the 3' LTR. The GUS cDNA was inserted in the NTK vector at the *Bgl* II site and in the NSV vector at the *Hind* III site. Bg, *Bgl* II; Bm, *Bam* HI; E, *Eco* RI; H, *Hind* III; X, *Xho* I.

3T3 cells. Clones producing high virus titers ($>1 \times 10^6$ cfu/ml; data not shown) were selected for further evaluation.

The ability of the vectors to encode enzymatically active GUS was determined using human and canine MPS VII fibroblast cell lines. The cells were infected with the recombinant viruses and G418-selected. The number of cells surviving in G418 indicated that the vector was transferred to $>95\%$ of the target cells (data not shown). More than 100 colonies were pooled to control for possible differences in expression due to different sites of integration and tested for GUS enzymatic activity.

Infection of the MPS VII fibroblasts with the NTK-BGEO vector produced by PA-317 clone 1 (TK-1 vector virus) and the NSV-BGEO vectors produced by PA-317 clones 2, 3, 5, and 8 (SV-2, SV-3, SV-5, and SV-8 vector viruses) resulted in the expression of normal or higher levels of GUS activity in human and canine MPS VII fibroblasts (Table 1). Infection of the MPS VII fibroblasts with the N2 vector, containing only the neo gene, had no effect on GUS activity (Table 1). The activity of IDUA, another lysosomal enzyme, was determined as a control in all experiments and was not changed by vector infection (Table 1).

To investigate the biological activity of the vector-encoded GUS enzyme on the natural substrates in diseased cells, primary cultures of normal and MPS VII canine RPE cells were used. In the retina, the RPE cells degrade large amounts of the proteoglycans located in the basal extracellular matrix and in the interphotoreceptor matrix that surround the visual cells (22). RPE cells were selected for functional studies because (i) primary cultures of a uniform population of cells could be evaluated rather than cell lines (23, 30); (ii) RPE cells are a differentiated cell type that is affected in MPS diseases, becoming hypertrophied from stored substrate (22); (iii) RPE cells have a specific pattern of synthesis, release, and degradation of GAGs (22, 30) against which specific changes in the GAG metabolic pathway can be evaluated; and (iv) RPE cells from MPS VII dogs retain the disease phenotype in tissue culture, storing chondroitin and heparan sulfates.

Primary cultures of RPE cells were established from normal and MPS VII dogs and infected with the vector viruses. Resistance to G418 indicated that the vectors were transferred to $>95\%$ of the target cells. Therefore, the comparisons between experimental groups were made on cells that had undergone approximately equal numbers of divisions. In the vector-treated cells, GUS activities were restored to the

Table 1. GUS activity in vector-treated MPS VII fibroblasts

Cell line	Vector	Enzymatic activity	
		GUS	IDUA
Normal human			
(MS-1)	None	27.5	1.5
(MS-2)	None	35.2	1.3
(MS-3)	None	18.7	0.9
(MS-4)	None	30.1	1.2
(MS-5)	None	41.5	0.7
MPS VII human (GM 151)	None	0.2	2.2
	None	0.5	1.8
	N2	0.4	2.8
	TK-1	74.4	1.9
	SV-2	412.0	3.6
	SV-3	122.0	2.7
	SV-5	144.0	4.1
	SV-8	381.0	1.4
MPS VII human (GM121a)	None	0.3	0.9
	N2	0.9	1.7
	TK-1	50.1	2.1
	SV-3	31.5	2.3
	SV-5	55.5	1.4
Normal dog (P33)	None	43.6	2.7
MPS VII dog (M165)	None	0.4	2.7
	N2	0.3	2.3
	TK-1	40.8	1.8
	SV-8	30.5	2.6
Normal rat (XC)	None	45.6	3.7

Vector designations represent the amphotropic vector virus produced by a clone of PA-317 packaging cells. All vector viruses infected >95% of target cells, as determined by G418 resistance. Enzyme activities are expressed as nmol of substrate cleaved per hr per mg of protein.

levels expressed in normal canine RPE cells with no change in IDUA activity (Table 2).

To determine whether the enzymes encoded by the vectors degraded the natural substrates in the MPS VII RPE cells, specific classes of GAGs were analyzed (30). A 72-hr labeling protocol was used that has been shown to measure the accumulation and turnover of GAGs in the lysosomal compartment and that clearly distinguishes normal from MPS cells (12, 31). In normal cells, newly synthesized GAGs reach equilibrium in the secretory pathway in 8–12 hr and in lysosomes by 24 hr (12, 31). In MPS cells, the secretory pathway is similar to normal cells, but radiolabeled GAGs continue to accumulate in lysosomes for at least 72 hr (12, 31). In canine MPS VII RPE cells, the stored GAGs in the dermatan sulfate/chondroitin sulfate fraction are mostly chondroitin sulfate, accounting for 75% of the excess GAGs, with heparan sulfate comprising the other 25% (unpublished

Table 3. GUS activity in vector-treated canine MPS VII bone marrow cells

Bone marrow source	Vector	Enzymatic activity		% cells staining positive for GUS	
		GUS	IDUA	Liquid culture	Pooled colonies
Normal	None	66.5	0.6	100	100
MPS VII	None	0.4	0.4	0	0
MPS VII	TK-1	78.6	0.4	9.4	32.5

Cells were harvested on day 12 (liquid culture) and day 13 (semisolid cultures) after infection. The cells tested had the staining characteristics (Giemsa) of hematopoietic cells. Enzymatic activities are expressed as nmol of substrate cleaved per hr per mg of protein. After staining for GUS activity, >100 cells were scored positive or negative in several fields by using Nomarski optics. Of the TK-1-infected MPS VII cells, 20 of 214 from liquid culture and 41 of 126 from semisolid culture stained positive. All TK-1-infected MPS VII cells grown in colony-forming cultures with G418 (0.8 mg/ml) to select for neo gene expression stained positive (data not shown).

results). In the cells treated with either the NTK-BGEO vector (TK-1) or the NSV-BGEO vector (SV-8), the precipitable radiolabeled GAGs in each fraction were reduced to the amounts found in the genetically normal RPE cells (Table 2). The GAGs precipitated from the culture medium of normal, MPS VII, and treated cells were equivalent (Table 2), confirming that the secretory pathway had reached equilibrium.

In lysosomal storage diseases, hematopoietic progenitor cells are possible targets for gene therapy (32). Therefore, canine MPS VII bone marrow cells were infected with culture supernatant containing a high titer (2×10^6 cfu/ml) of vector virus and tested for enzyme expression in long-term cultures. The vector-infected MPS VII hematopoietic cells apparently expressed normal amounts of GUS enzymatic activity (Table 3). This was an unexpected result because retroviral vector transfer of the neo gene to canine bone marrow cells usually has been found to be inefficient when assayed for G418-resistant colony formation (33–35). To determine if the vector-encoded enzyme was present in all of the vector-treated bone marrow cells, histochemical staining for GUS enzymatic activity (28, 29) was used to visualize individual cells in infected populations (Fig. 2). As positive controls, XC cells and TK-1-corrected GM-151 cells, which expressed normal levels of GUS activity in the quantitative assay (Table 1), stained positively. The histochemical reaction product was present in a granular pattern in the cytoplasm (Fig. 2 A and D), which represents lysosomal localization of GUS enzymatic activity (28, 29). The uninfected and the N2-infected GM-151 cells were negative in both assays (Table 1 and Fig. 2 B and C).

The percentage of GUS-positive hematopoietic cells in cultures of vector-treated MPS VII bone marrow cells was

Table 2. Effect of GUS correction on substrate concentrations in primary canine MPS VII RPE cells

RPE cell source	Vector virus	Enzymatic activity		Precipitable GAGS			
		GUS	IDUA	Cell layer		Medium	
				HS	DS/CS	HS	DS/CS
Normal	None	48.5 ± 17.2	23.0 ± 2.8	424 ± 235	3087 ± 667	144 ± 31	3411 ± 483
MPS VII	None	0.1 ± 0.2	20.3 ± 1.8	2543 ± 476	7634 ± 345	130 ± 8	3106 ± 59
MPS VII	TK-1	34.2 ± 10.0	19.4 ± 5.5	462 ± 92	2357 ± 209	133 ± 19	3154 ± 55
MPS VII	SV-8	49.5 ± 29.0	27.2 ± 6.9	815 ± 222	1860 ± 483	122 ± 11	2266 ± 105

Treated and untreated MPS VII RPE cells were from the same animal. Each point represents data from five samples (mean ± 1 SD). Values for enzymatic activity are expressed as nmol of substrate cleaved per hr per mg of protein. All of the cultures contained similar amounts of total protein (data not shown). The values for GAG incorporation are expressed as dpm of $^{35}\text{SO}_4$, adjusted for equal amounts of protein (mean ± SD). DS, dermatan sulfate; CS, chondroitin sulfate; HS, heparan sulfate. Radiolabeled bands that comigrated with dermatan sulfate and chondroitin sulfate ran too close together to separate and were measured together (30). The identity of each GAG was confirmed by enzymatic degradation and deamination (30), which showed that 75% of the excess GAG was chondroitin sulfate and 25% was heparan sulfate (unpublished results).

FIG. 2. Histochemical staining of normal, MPS VII, and vector-corrected MPS VII cells for GUS enzymatic activity. (A) Rat XC cells. (B) Uninfected human MPS VII fibroblasts (GM-151 cells). (C) GM-151 cells infected with the N2 vector and G418-selected. (D) GM-151 cells infected with the TK-1 vector and G418-selected. (E) Normal canine bone marrow cells. (F) Uninfected canine MPS VII (dog M150) bone marrow cells. (G) M150 bone marrow cells infected with the TK-1 vector. GUS activity is present (red stain) in the normal cells (A and E) and in the vector-corrected MPS VII cells (D and G). No GUS activity is present in the untreated MPS VII cells (B and F) or in the N2-infected cells (C). The bar shown in A represents 60 μ m in A, B, and D; 100 μ m in C; and 25 μ m in E-G.

determined from the proportion of stained and unstained cells. In cultures that expressed normal amounts of GUS activity (Table 3), only $\approx 10\%$ of the cells stained positively (Table 3), and individual cells stained more intensely than normal bone marrow cells (Fig. 2, G compared to E; F shows uninfected MPS VII cells). Thus, the apparent normalization of GUS activity in these cultures was due to higher than normal activity in a fraction of the cells tested. This indicated that individual transduced cells expressed high levels of the vector-encoded enzyme even though the efficiency of transduction of cells exposed to vector virus infection was low. The transduction efficiency was also determined on vector-treated cells that grew in colony-forming cultures because

vector integration is dependent upon cell division. Only one-third of the cells from pooled colonies were GUS-positive (Table 3), indicating that even among cells undergoing mitosis, a substantial fraction were refractory to vector transduction. The low rate of transfer occurred despite the use of high-titer vector virus preparations that infected virtually all of the MPS VII fibroblasts and RPE cells.

DISCUSSION

Our results demonstrate that the rat GUS cDNA, driven by either the thymidine kinase or SV-40 promoter, expresses enzymatically active GUS at normal or higher levels when

transferred by retroviral vectors into a number of cell types from human and canine patients with MPS VII. As is usual in such studies, the levels of enzymatic activities in the cells were assayed in cell homogenates using artificial substrates (25, 26). However, the catalytic activity of lysosomal enzymes toward artificial substrates can be markedly different than toward the natural substrates (36, 37). The cDNA-encoded enzyme should reach the lysosomal compartment since the signals for cellular sorting of lysosomal enzymes are contained in the coding region (16, 38, 39), but proof that the vector-encoded enzyme is catalytically active toward the stored substrates in the lysosomal compartment of diseased cells can only be obtained by direct measurements of degradative function. Retroviral transfer of a human glucocerebrosidase cDNA has been shown to restore the enzymatic activity in cultured fibroblasts and lymphocytes from patients with type I Gaucher disease, but correction of the degradative defect could not be shown in these cell types because the phenotypic cellular defect in this disease is manifest only in the macrophage lineage (4).

We investigated the efficacy of the vector-encoded enzyme in reversing the degradative defect in a differentiated cell type that is severely affected in MPS VII and in which specific changes in the GAG metabolic pathway could be evaluated: canine RPE cells. These studies clearly show that the defect in degradation of specific GAGs in the MPS VII cells is corrected after vector infection, providing strong evidence that the vector-encoded GUS is normally sorted to and active within lysosomes. The pattern of cytoplasmic localization of GUS activity in treated cultured MPS VII bone marrow cells is also compatible with normal lysosomal localization and activity of the enzyme in hematopoietic cells (28, 29).

These vectors may be useful for *in vivo* gene transfer studies in the canine and murine models of MPS VII (17, 18). The most promising target tissue for gene transfer in MPS VII and other lysosomal storage disorders appears to be the hematopoietic cells of the bone marrow (32). This strategy depends on the export of lysosomal enzymes from corrected hematopoietic cells to other diseased cells of the patient (cross-correction) (31). However, it will be necessary to overcome the problem of maintaining long-term expression of retroviral-vector-transferred genes in hematopoietic cells *in vivo* (2). The *in vitro* experiments described in this report indicate that GUS can be expressed at high levels in hematopoietic cells. However, histochemical visualization of expression of the transferred gene in individual cells indicated that the vectors either did not efficiently infect the bone marrow cells, even when they were dividing, or that only a fraction of infected cells expressed the vector-transferred gene.

Although retroviral vector transduction of hematopoietic stem cells has been accomplished (40, 41), it will be necessary to achieve efficient transduction of a high percentage of these cells for this strategy to be effective in individual patients. An alternative approach for lysosomal storage diseases may be to transplant genetically corrected fibroblasts as a source of normal enzyme (2) that could circulate to other affected cells. The MPS VII model may be a useful test system for this approach since the vector-corrected MPS VII fibroblasts release active GUS into culture supernatants (unpublished results) and GUS is a very stable enzyme (42).

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