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Various Cells Retrovirally Transduced with *N*-Acetylgalactosoamine-6-Sulfate Sulfatase Correct Morquio Skin Fibroblasts *In Vitro*

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ABSTRACT

Gene therapy may provide a long-term approach to the treatment of mucopolysaccharidoses. As a first step toward the development of an effective gene therapy for mucopolysaccharidosis type IVA (Morquio syndrome), a recombinant retroviral vector, LGSN, derived from the LXSN vector, containing a full-length human wild-type *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) cDNA, was produced. Severe Morquio and normal donor fibroblasts were transduced by LGSN. GALNS activity in both Morquio and normal transduced cells was several fold higher than normal values. To measure the variability of GALNS expression among different transduced cells, we transduced normal and Morquio lymphoblastoid B cells and PBLs, human keratinocytes, murine myoblasts C2C12, and rabbit synoviocytes HIG-82 with LGSN. In all cases, an increase of GALNS activity after transduction was measured. In Morquio cells co-cultivated with enzyme-deficient transduced cells, we demonstrated enzyme uptake and persistence of GALNS activity above normal levels for up to 6 days. The uptake was mannose-6-phosphate dependent. Furthermore, we achieved clear evidence that LGSN transduction of Morquio fibroblasts led to correction of the metabolic defect. These results provide the first evidence that GALNS may be delivered either locally or systematically by various cells in an *ex vivo* gene therapy of MPS IVA.

OVERVIEW SUMMARY

Gene therapy is actively being considered for the treatment of mucopolysaccharidoses and other lysosomal diseases. The importance of evaluating this approach is emphasized by the lack of any effective alternative. Here, we show retrovirus-mediated gene correction of MPS IVA (*N*-acetylgalactosamine-6-sulfate sulfatase deficiency) fibroblasts and the transfer of GALNS from retrovirally transduced cells to Morquio cells.

INTRODUCTION

MUCOPOLYSACCHARIDOSIS TYPE IVA (MPS IVA) is an autosomal inherited disorder characterized by a deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS; E.C. 3.1.6.4) (Singh *et al.*, 1976). Clinically MPS IVA can be divided into several subtypes: the severe (so-called Morquio disease), an intermediate form and a mild form (Beck *et al.*, 1986). Severely affected patients do not survive beyond their twenties or thirties, although this autosomal recessive disorder exhibits

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a wide spectrum of clinical heterogeneity (Glössl *et al.*, 1981). Full-length cDNA for human GALNS has been cloned and sequenced (Tomatsu *et al.*, 1991) and several alterations have been discovered (for review, see Tomatsu *et al.*, 1997), including point mutations in exons and in splice sites in genes from MPS IVA patients. A structural molecular model of GALNS was recently proposed revealing effects of different mutations on protein function (Sukegawa *et al.*, 2000). GALNS deficiency causes accumulation of keratan sulfate and chondroitin-6-sulfate within lysosomes. As a consequence, patients affected by MPS IVA have typical features including specific spondyloepiphyseal dysplasia, short trunk dwarfism, coxa valga, odontoid hypoplasia, corneal opacities, and excessive urinary excretion of keratan sulfate and chondroitin-6-sulfate although intelligence is preserved (Northover *et al.*, 1996).

Conventional therapy is symptomatic and limited to palliative procedures, which have virtually no impact upon mortality. Therefore, gene therapy could represent a great therapeutic improvement (Salvetti *et al.*, 1995a). Because lysosomal enzymes can be taken up by deficient cells using the mannose-6-phosphate pathway (Dahms *et al.*, 1989), strategies aimed at providing cellular source of enzymes have been tried. For example, bone marrow transplantation (BMT) has now been performed on a significant number of MPS patients, but there is still no general consensus about the effectiveness of this treatment (Clink and Ozand, 1992; Hoogerbrugge *et al.*, 1995). The clinical result of BMT in the MPS patients has varied considerably; factors that affect the outcome include the type of the MPS disorder, the donor genotype, the preparative regimen, the degree of clinical involvement, and the age at the time of transplantation (Neufeld and Muenzer, 1995). Even under the best conditions, benefits of BMT are modest, while the risks and the cost of the procedure are great (Neufeld, 1991). However, its limited success has encouraged the development of gene replacement through transfection of hematopoietic progenitor cells. Nevertheless, current transduction efficiency of human hematopoietic stem cells using retroviral vectors needs to be increased further before gene transfer can be used for therapeutic applications (Kiem *et al.*, 1995; Simonaro *et al.*, 1999).

Here, we report the first *in vitro* preclinical data on gene therapy of MPS IVA. We evaluate a possible systemic enzyme delivery approach mediated by retrovirally transduced cells of different origin as well as local therapy to joints by injection of retrovirally transduced synoviocytes.

MATERIALS AND METHODS

Cell lines

Murine ecotropic packaging cell lines GP+E-86 (Markowitz *et al.*, 1988a), amphotropic GP+*env*Am12 (Markowitz *et al.*, 1988b), human fibroblasts from healthy donor and Morquio patients (kindly provided by Dr. R Gatti, Gaslini Institute, Genoa, and Dr. MT Zabot, Hopital Debrousse, Lyon, France), were cultivated in Dulbecco's Eagle's modified medium (DMEM) (Hyclone, Logan, UT) containing 10% (vol/vol) fetal calf serum (FCS) (GIBCO-BRL, Gaithersburg, MD). C3HC2C12 (C2C12) myoblasts were also grown in DMEM containing 10% FCS and induced to differentiate by shifting culture medium to DMEM containing 2% FCS. Peripheral blood lymphocytes (PBLs) from normal donors and a MPS IVA patient were separated by Ficoll-Hypaque (Amersham Pharmacia Biotech) gradient and grown under phytohemagglutinin (PHA) (2 μg/ml) and human interleukin-2 (hu-rIL-2) (100 u/ml) stimulation in Iscove's modified Dulbecco's medium (IMDM) containing 10% FCS or RPMI containing 5% human serum. Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines established from a normal donor and a Morquio patient (kindly provided by Dr. CK Stein, SUNY Health Science Center, Syracuse, NY) were grown in DMEM containing10% FCS. Normal human keratinocytes were obtained from healthy donors and cultivated on a lethally irradiated feeder-layer of 3T3 cells as previously described (Green *et al.*, 1979). Rabbit synoviocytes HIG-82 (Georgescu *et al.*, 1988) from the American Type Cell Culture Collection (ATCC CRL-1832, Rockville, MD) were grown in Ham's F12 (Sigma, St Louis, MO) medium containing 10% FCS.

Vector construction

The 1.6-kb *Eco* RI–*Eco* RI fragment containing the entire human GALNS cDNA (Tomatsu *et al.*, 1991) was inserted in the *Eco* RI site of the vector LXSN (Miller and Rosman, 1989) to generate the recombinant vector LGSN. All plasmids were grown in *Escherichia coli* strains DH5α and JM 101. The mock control SFCMM-2 vector (Bonini *et al.*, 1997) codes for a truncated form of the low-affinity human nerve growth factor receptor, and for the HSV-Tk/neo fusion protein, a bifunctional protein carrying both the HSV-Tk activity and the neomycin resistance.

Virus production

Plasmid LGSN was transfected into ecotropic packaging cell line E-86 by calcium phosphate co-precipitation. After 48 hr, culture medium was harvested and filtered through a 0.20-μm filter (Nalgene, Rochester, NY) and used to transduce Am12 amphotropic packaging cell line. Twenty-four hours after transduction, Am12 cells were subcultured 1:10 into medium containing 0.8 mg/ml G418 (Geneticin, Roche, Mannheim, Germany). After about 10 days, individual colonies were picked, expanded, and used for production of virus stocks. Individual G418 colonies were screened for virus titer by transduction of NIH 3T3 cells and level of GALNS activity in cell extracts.

Retroviral gene transfer

Fibroblast transduction: Skin fibroblasts were cultivated in DMEM containing10% FCS at 30% confluence in a 25-cm^2 flask 24 hr before transduction. Cells were incubated for about 1 hr with 3 ml of the viral supernatant containing 8 μg/ml Polybrene with intermittent shaking; an additional 3 ml of medium and 8 μg/ml Polybrene were added and the cells were returned to the incubator for 18 hr. After 48 hr transduced cells were selected by addition of 0.7 mg/ml G418.

Transduction of lymphoblastoid cell lines: EBV-lymphoblastoid B cells were transduced by co-cultivation with retrovirus producing irradiated (10,000 rads) cells for 72 hr in DMEM containing 10% FCS in the presence of 8 μg/ml Polybrene.

Transduction of human PBLs: PBLs obtained from fresh blood samples were grown for 72 hr under PHA and h-IL2 stimulation in IMDM containing 10% FCS or RPMI containing 5% human serum. Viral transduction was performed by co-cultiva-

tion with irradiated virus-producing cells in DMEM containing 10% FCS and 8 μg/ml Polybrene. After 72 hr, PBLs were harvested and selected in 0.7 mg/ml G418.

Transduction of human keratinocytes: Primary human keratinocytes obtained from human healthy skin samples were seeded (3×10^4 cells/cm^2) onto a feeder-layer (2.3×10^4 cells/cm^2) composed of lethally irradiated (6,000 rads) 3T3 cells and retrovirus-producing cells (a 1:2 mixture). Epidermal growth factor (10 ng/ml) was added at plating. Subconfluent cultures were then passaged and cultured to confluence in SFM medium (GIBCO-BRL) without a feeder layer. Keratinocytes were subjected to no selective agents after transduction.

Transduction of synoviocytes: Rabbit synoviocytes HIG-82 were from the ATCC (CRL-1832). Cells were grown to about 75% confluence in a 25-cm^2 flask containing 4 ml of Ham's F12 medium and 10% FCS. Medium was then removed and cells were transduced by adding 1 ml of retroviral supernatant (titer of 10^6 CFU/ml) in presence of 8 μg/ml Polybrene. After 2 hr of incubation with intermittent shaking, an additional 3 ml of medium was added and the cultures were returned to the incubator for 72 hr. Cultures were treated with trypsin and reseeded at 1:5 split ratio. Cells were then selected in 0.5 mg/ml G418.

Transduction of C3HC2C12 (C2C12) myoblasts: C2C12 myoblasts were grown to about 50% confluence in a 25-cm^2 flask containing 4 ml of DMEM and 10% FCS. Medium was then removed and cells were transduced by adding 2 ml of retroviral supernatant (titer of 10^6 CFU/ml) in the presence of 8 μg/ml Polybrene. After 2 hr of incubation with intermittent shaking, an additional 3 ml of medium was added and the cultures were returned to the incubator for 16 hr. Cultures were treated with trypsin and reseeded at 1:10 split ratio. After 24 hr, cells were then selected in 1.0 mg/ml G418.

Synthesis of 4-methylumbelliferyl-β-D-galactopyranoside-6-sulfate

4-Methylumbelliferyl-β-D-galactopyranoside-6-sulfate (MU-Gal-6S) was synthesized by sulphation of 4-methylumbelliferyl-β-D-galactopyranoside (Sigma, St. Louis, MO), by analogy with the method used by Bayleran, to synthesize 4-methylumbelliferyl-β-D-6-sulfo-N-acetylglucosaminide (Bayleran *et al.*, 1984).

Determination of GALNS activity

Cells were harvested, washed in PBS, lysed by a freeze–thaw cycle followed by 5 min of sonication (Branson, Soest, NL, mod. 1210) in distilled water and then clarified by microcentrifugation. A 100-μl aliquot of cell extract was diluted to 2 ml in water and reconcentrated in a Centricon 30 microconcentrator (Millipore, Bedford, MA). This step was repeated three times. GALNS activity was determined as follows. Briefly, 3–20 μg of total protein was incubated in a reaction mixture (30 μl total volume) containing 18 μl of 22 m*M* MU-Gal-6S in 0.1 *M* NaCl, 0.1 *M* sodium acetate (pH 4.3). After incubation for 18 hr at 37°C, 2 μl of a solution containing 10 mg/ml of β-galactosidase from *Aspergillus orizae* (Sigma, St Louis, MO) in 0.1 *M* NaCl, 0.1 *M* sodium acetate (pH 4.3) were added, and the samples incubated for additional 2 hr at 37°C. The reaction was stopped adding 970 μl of 0.5 *M* glycine buffer (pH 10.5) and analyzed in a fluorimeter (Jasco, Tokyo, Japan, mod. FP 777; λ_{ex} 366, λ_{em} 450 at 25°C). The procedure adopted differs from that reported (van Diggelen *et al.*, 1990) in that: (1) substrate concentration that was about 20-fold higher in our test, with a substantial improvement in sensitivity and reproducibility; (2) *Aspergillus orizae* β-galactosidase instead of Morquio cell extracts was added. A unit of enzyme activity corresponds to the release of a 1 nmol of 4-methylumbelliferone per minute. Protein content was assayed with the Bradford (Pierce, Rockford, IL) reagent using BSA as standard.

Other enzyme assays

β-Glucosidase and arylsulfatase A were assayed according to the methods described in Daniels and Glew (1982) and Fluharty and Edmonds (1978), respectively.

Secretion of GALNS

To determine whether the recombinant GALNS is released in an active form in the culture media, an equal number of normal, Morquio, and retrovirus-transduced Morquio fibroblasts were cultured in reduced serum medium Opti-MEM (GIBCO-BRL). After 3 days of culture, medium was collected, filtered through a 0.22-μm filter to remove cells and concentrated in a Centricon 30. The total amount of GALNS activity was assayed.

Evaluation of GAGs accumulation

Selected populations of transduced Morquio fibroblasts and unmanipulated normal fibroblasts were grown in $MgSO_4$-defective medium (MEM Gibco, Life Technologies, Milan, Italy) supplemented with 10% dialyzed FCS. After 72 hr, the cells were trypsinized and plated in the presence of 4 μC/ml of $Na_2{}^{35}SO_4$. Cells were harvested daily for 4 days, washed with PBS, and lysed by multiple cycles of freeze–thaw. The extent of radioactive sulfate incorporation was assessed essentially as reported by Di Francesco *et al.* (1997) with minor modifications. Briefly, after lysis, 1 ml of 80% ethanol was added to cell samples. After boiling for 5 min, they were centrifuged at $5,000 \times g$ for 3 min and the pellets washed twice as above using 2 ml of 80% ethanol. Then, 0.25 ml of 10% NaOH was added and samples were heated at 100°C for 5 min; 0.20-ml aliquots were withdrawn and radioactivity determined by liquid scintillation counting in Ultima Gold (Packard Instruments, Meriden, CT). Prior to ethanol addition, the protein content of each sample was assayed with the Bradford (Pierce, Rockford, IL) reagent, using BSA as standard.

Co-culture experiments

Co-culture experiments were performed in 75-mm Petri dishes or 24-mm transwells in which the two chambers are separated by a 0.4-μm pore membrane (Costar, Cambridge, MA).

RESULTS

Construction of recombinant retrovirus LGSN and generation of a high-titer producer cell line

The 1.6-kb *Eco* RI cDNA fragment encoding GALNS from pCAGGS (Tomatsu *et al.*, 1991) was ligated into the *Eco* RI site of the retroviral vector LXSN, thus placing GALNS cDNA sequence under the transcripitional control of the Moloney leu-

kemia virus long terminal repeat (MoMLV-LTR). This vector, LGSN, was used for transfection of E-86 cells. Virus rescued from transfected E-86 was used to transduce Am12 cells, and 20 clonal cell lines were isolated and analyzed for titer (G418-resistant colony-forming units), GALNS activity, and GALNS cDNA integration, as assessed by Southern blot analysis (data not shown). Titers ranged from 3×10^4 to 7×10^5 CFU; GALNS activity ranged from 6- to 10-fold with respect to the mock control in the highest titer clones. The clones producing highest viral titer were tested for production of helper virus and were found to be negative.

Transduction of different cell lines

Because MPS IVA clinical manifestations involve many different organs, a careful consideration needs to be given to the choice of the cells to be genetically modified and used as a source of the deficient enzyme. Therefore, we evaluated GALNS activity before and after retroviral transduction in several cell lines.

In particular, we analyzed transduced fibroblasts, PBLs, keratinocytes, muscle cells, and synoviocytes for the production of GALNS and their ability to secrete the enzyme or cross-correct negative cells in co-culture experiments.

Fibroblasts: Autologous fibroblasts (which can be easily obtained from skin biopsy) grown *in vitro* for retrovirus-mediated gene transfer and reimplanted into intraperitoneal implants (neo organs) might represent one convenient source of genetically modified material for *in vivo* delivery of missing enzymes (Salvetti *et al.*, 1995b; Yogalingam *et al.*, 1999).

We assayed GALNS activity in cell extracts of normal and patients' fibroblasts before and after transduction with the LGSN or the mock control SFCMM-2 vectors and selection in medium containing G418 (Table 1). An increase of GALNS activity of about 50-fold with respect the normal value was revealed in both normal and Morquio-transduced fibroblasts. To confirm that the detected activity depended on GALNS, several measurements were performed at different pH values. The pH activity profile matched that previously reported for GALNS, with an optimal activity at pH 4.3 (Fig. 1) (van Diggelen *et al.*, 1990).

To determine whether recombinant GALNS was released from transduced cells, normal, Morquio, and retroviryus-transduced Morquio fibroblasts were cultured in reduced serum medium, as described in the Materials and Methods section. Total amount of GALNS activity was undetectable in medium from Morquio fibroblasts culture and about 40 times higher than normal values medium from Morquio transduced fibroblasts (data not shown).

The expression of GALNS in transduced cells had no significant effect on the level of the other lysosomal enzymes activities assayed (β-glucosidase, arylsulfatase A) (data not shown).

Peripheral blood lymphocytes and lymphoblastoid B cell lines: Ex vivo lymphocyte gene therapy was used in a phase I/phase II clinical protocol for the treatment of mild Hunter syndrome (MPS type II) (Whitley *et al.*, 1996). Cell transplantation of lymphocytes may correct a population of cells and enzyme replacement of negative cells may occur, if sufficient enzyme is released from the transduced lymphocytes. More-

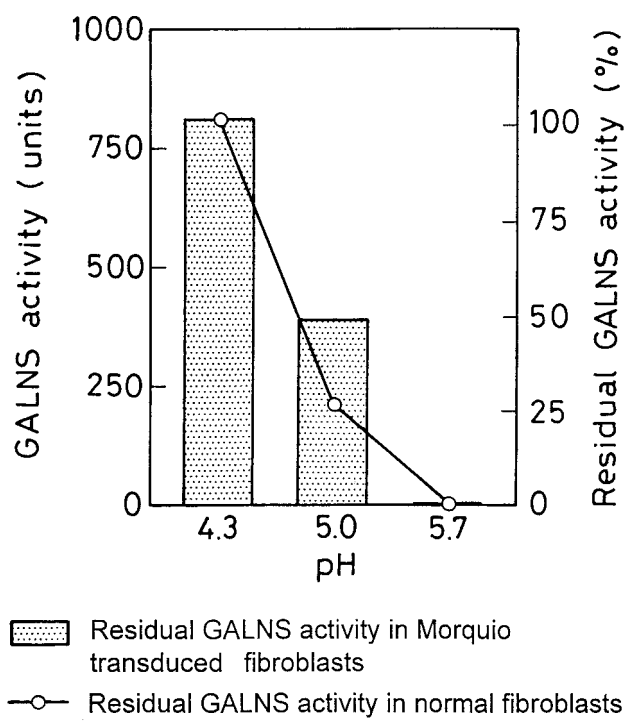

FIG. 1. pH profile of GALNS activity in Morquio transduced fibroblasts (bar graph) compared with the percentage of residual GALNS activity in normal fibroblasts at different pH as described in van Diggelen *et al.* (1990) (line chart).

over, although PBLs may not be able to secrete much lysosomal enzymes after transfection, (Bou-Gharios *et al.*, 1993; Sangalli *et al.*, 1998), they circulate widely, and enzyme transfer by cell-to-cell contact may also play a role in enzyme replacement to lysosomes (Olsen *et al.*, 1981; Bou-Gharios *et al.*, 1993).

To evaluate such an approach we transduced PBLs and lymphoblastoid B cells (EBV cell lines) as an *in vitro* model of blood circulating cells. PBLs from healthy donors, a Morquio patient and a Morquio carrier were transduced with the vector carrying the GALNS cDNA or the mock control. GALNS activity was assayed in cell extracts before and after transduction and G418 selection (Table 1); GALNS activity in Morquio PBLs rose from a nearly undetectable level to a level comparable with the carrier. EBV cell lines established from a healthy donor and from a MPS IVA patient were transduced with the LGSN or the mock control SFCMM-2 retroviral vectors. GALNS activity in Morquio EBV cell extracts after retroviral transduction without G418 selection was about five-fold higher than the normal value, and about 30-fold higher than the Morquio EBV nontransduced cells (Table 1).

Human keratinocytes: The epidermis is an attractive site for therapeutic gene delivery because it is accessible and capable of delivering gene products to the systemic circulation (Choate and Khavari, 1997). Moreover, keratinocytes exhibit features that make them a suitable target for *ex vivo* gene transfer, namely the presence of stem cells (Barrandon and Green, 1987) and a high rate of transduction by retroviral vectors (Mathor *et al.*, 1996). Grafts of autologous keratinocytes genetically altered to secrete a new gene product are, therefore, a potential vehicle for gene therapy for inherited diseases (Fenjves *et al.*, 1994).

Human keratinocytes established from skin samples of a healthy donor were transduced with the LGSN or the mock control SFCMM-2 retroviral vectors. GALNS enzyme activity was assayed in cell extracts before and after transduction with retroviral vectors without G418 selection (Table 1). After transduction, GALNS activity was increased of about 50-fold compared to the normal level.

C3HC2C12 (C2C12) myoblasts: The delivery of a recombinant protein from genetically modified muscles has been documented by engrafting immortalized murine C3HC2C12 (C2C12) myoblasts in adult animals (Barr and Leiden, 1991). The main advantage is that myofibers are highly vascularized and long-lasting structures that are easily accessible for *in vivo* and *in vitro* manipulation. Moreover, C2C12 cells are used as long-term enzyme-producing, encapsulated xenogenic cell lines for the treatment of neurodegenerative diseases (Déglon *et al.*, 1996).

We transduced C2C12 with the LGSN or the mock control SFCMM-2 retroviral vectors and assayed GALNS enzyme activity in cell extracts before and after transduction and G418 selection (Table 1). We also measured GALNS activity in C2C12 myoblasts induced to differentiate by shifting culture medium to low serum after selection. We found an increase of 33-fold in myoblasts and 35-fold in myotubes, compared with the level of GALNS activity before retroviral transduction.

HIG-82 synoviocytes: For local intraarticular gene delivery, the synovium is an attractive target because it is readily accessed and is in direct contact with the joint space. The synovial cells may be grown *in vitro*, transduced with retroviral vectors carrying genes of interest and then returned to joints by intraarticular injection (Bandara *et al.*, 1992). The autografted, transduced synoviocytes are captured by the synovium, where they continue to express their transgenes. Moreover, direct intraarticular injection of retroviral vectors can be used for *in vivo* gene delivery to the synovial lining cells of joints (Ghivizzani *et al.*, 1997). We studied GALNS expression in rabbit synoviocytes HIG-82 transduced with the LGSN retroviral vector or the mock control and selected in G418 (Table 1). GALNS activity was about five-fold higher than before retroviral transduction.

TABLE 1. GALNS ACTIVITY IN DIFFERENT CELL LINES (EXPRESSED IN UNITS/MG OF TOTAL PROTEINS)

Cell line	*Normal*	*Mock*[a]	*LGSN*
Normal fibroblasts[b]	17.2 ± 5.8	23.7 ± 0.4	894 ± 86
Morquio fibroblasts[b]	0.36 ± 0.09	0.30 ± 0.11	842 ± 32
Normal EBV	1.10 ± 0.35	1.03 ± 0.01	21.1 ± 6.4
Morquio EBV	0.19 ± 0.09	n.a.	5.91 ± 0.08
Normal PBLs	4.73 ± 0.64	5.46 ± 0.21	22.7 ± 0.4
Carrier PBLs	0.85 ± 0.03	n.a.	7.02 ± 0.13
Morquio PBLs	range: b.d.–10.03	n.a.	2.51 ± 0.12
Human keratinocytes	4.8 ± 0.22	6.25 ± 0.55	258 ± 1
C2C12 murine myoblasts	16.2 ± 6.2	16.5 ± 1.0	529 ± 70
C2C12 murine myotubes	n.a.	31.3 ± 0.2	1080 ± 100
HIG 82 rabbit synoviocytes	56.9 ± 31.5	51.7 ± 31.1	313 ± 141

b.d., Below detection limit; n.a., not assayed.

All data are shown as average enzyme activity of at least quadruplicate samples ± standard deviation.

[a]SFCMM-2 transduced.

[b]Assayed on two different human normal and Morquio skin fibroblast cell lines.

Co-cultivation of Morquio fibroblasts and retroviral transduced cell lines: An *in vitro* model for enzyme replacement by somatic cell gene transfer was developed to examine the capacity of GALNS transduced cells to cross-correct cells from MPS IVA patients. A total of 1×10^6 MPS IVA-transduced fibroblasts were co-cultured in transwell dishes with 1×10^6 Morquio fibroblasts; after 72 hr of co-culture, transduced cells were removed and Morquio recipient cells were washed in PBS and cultured in fresh medium for an additional 3 or 5 days. Morquio fibroblasts were able to take up enzyme from the medium of culture and then maintained a GALNS activity level up to normal for at least 5 days after the end of co-culture (Table 2). Uptake was nearly completely inhibited in presence of 5 m*M* D-mannose 6-phosphate. These data are supported by experiments of cross-correction of Morquio fibroblasts cultured in 0.2-μm filtered medium previously used to cultivate transduced cells for 24 hr (data not shown).

In addition, 1×10^6 MPS IVA fibroblasts were co-cultured in transwell dishes with 1.6×10^7 EBV-transduced cells. After 72 hr of co-culture transduced cells were removed and GALNS activity was assayed in cell extracts. There was an increase of GALNS activity of about six-fold in Morquio fibroblasts (Table 2). Similarly, we performed cross-correction experiments between retrovirus-transduced PBLs and Morquio fibroblasts; no significant increase of GALNS activity in the negative cells was seen (Table 2), confirming that lymphocytes secrete only barely detectable levels of lysosomal enzymes due to their inability to secrete high levels of enzyme bearing the proper modification for receptor-mediated cellular uptake (Bou-Gharios *et al.*, 1993).

Transduced keratinocytes, C2C12, and HIG-82 cells were able to cross-correct human Morquio fibroblasts co-cultivated in transwell dishes in a 1:1 ratio (Table 2). In particular, the level of GALNS activity in Morquio fibroblasts after co-culture with transduced keratinocytes was comparable to the level of normal fibroblasts (Table 1), with a 50-fold increase with respect to the basal level of Morquio fibroblasts. The level of GALNS activity in Morquio fibroblasts after co-culture with transduced muscle cells was about 15-fold the level of normal fibroblasts (Table 1). Moreover, the GALNS activity level increased of about 20-fold in Morquio fibroblasts after co-culture with transduced HIG-82 synoviocytes. Co-culture with normal and mock-transduced cells does not lead to any significant increase of GALNS activity in Morquio fibroblasts in all the analyzed cell lines (data not shown).

Stability of recombinant enzyme

Both the drug delivery and the local joint approach presuppose secretion of recombinant GALNS into the bloodstream or synovial fluid and its uptake by deficient cells by way of the mannose-6-phosphate pathway to the lysosomes. To check the feasibility of such approaches, we evaluated the stability of the enzyme under physiological conditions. Recombinant GALNS retained at least 70% of its catalytic activity after 2 hr of incubation in human plasma or synovial fluid at 37°C (Fig. 2).

Metabolic correction of transduced Morquio fibroblasts

To assess whether the GAG storage defect found in Morquio cells could be relieved by transfection with the LGSN vector, G418-selected populations of Morquio fibroblasts were preincubated with $^{35}SO_4$; the extent of GAG accumulation was determined from day 2 to day 4, as reported (Di Francesco *et al.*, 1997). In parallel, normal and SFCMM2-transduced Morquio fibroblasts were assayed. Fibroblasts from 2 Morquio patients were analyzed and yielded reproducible results. Data from one of them are shown in Fig. 3. LGSN transduction completely abolished the accumulation of GAGs in Morquio cells, as shown by GAG levels dropped to values close to or even lower than those of normal cells (Fig. 3). This provides clear evidence that LGSN transduction does correct the GAG storage defect in Morquio fibroblasts.

TABLE 2. GALNS ACTIVITY IN MORQUIO FIBROBLASTS AFTER CO-CULTURE WITH DIFFERENT TRANSDUCED CELL LINES (EXPRESSED IN UNITS/MG OF TOTAL PROTEINS)

Co-culture cell line	*GALNS activity in Morquio fibroblasts after co-culture*[a]
Morquio fibroblasts LGSN	193 ± 3
Morquio fibroblasts LGSN + 5 m*M* mannose 6-phosphate	2.20 ± 0.34
Morquio fibroblasts LGSN 3 days after end of co-culture	142 ± 6
Morquio fibroblasts LGSN 5 days after end of co-culture	81.5 ± 5.9
EBV LGSN	2.04 ± 0.03
PBLs LGSN	0.77 ± 0.02
Keratinocytes LGSN	20.8 ± 1.9
C2C12 murine myoblasts LGSN	309 ± 35
HIG 82 rabbit synoviocytes LGSN	8.01 ± 1.46

All data are shown as average enzyme activity of at least quadruplicate samples ± standard deviation.

[a]Morquio fibroblasts, GALNS basal level is 0.36 ± 0.09.

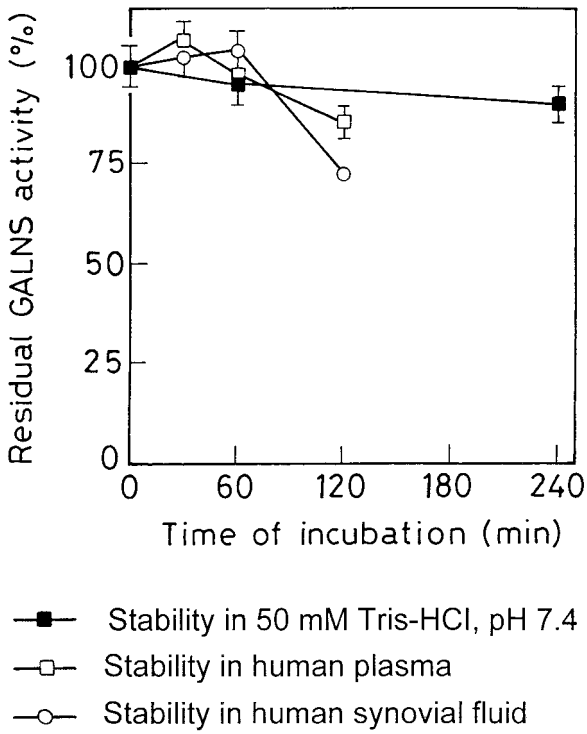

FIG. 2. GALNS stability profile in 50 m*M* Tris-HCl (pH 7.4) in human plasma and human synovial fluid.

DISCUSSION

Newly synthesized lysosomal enzymes are able to circulate from cell to cell (Olsen *et al.*, 1981). This process is mediated by to the well-characterized mannose-6-phosphate signal/receptor system, which is responsible for the efficient endocytosis of soluble lysosomal enzymes as well as a proper subcellular localization of both endocytosed and intracellular enzymes (Dahms *et al.*, 1989). Therefore, treatment of only a relatively small number of cells expressing high level of GALNS could provide the enzyme source for the correction of the lysosomal metabolism in target organs in Morquio patients. Here we describe an *ex vivo* model of gene therapy for mucopolysaccharidosis type IVA that provides the first evidence that GALNS may be efficiently delivered systemically or locally from retrovirally transduced cells of various origin to Morquio cells.

Studies *in vitro* have shown that enzyme-deficient patients' cells can be corrected by an exogenous supply of the missing enzyme (Neufeld and Muenzer, 1995). Transient improvement of patient conditions have been obtained with leukocyte and plasma infusions, whereas fibroblast transplantation has not been successful in restoring any significant enzyme activity. BMT has been suggested as a potential method for enzyme supplement for MPS patients, but the effectiveness of BMT still has to be investigated (Hoogerbrugge *et al.*, 1995). Recent studies on a feline model of MPS VI clearly demonstrated an excellent clinical response to enzyme replacement therapy, particularly in preventing or considerably reducing bone pathologies (Crawley *et al.*, 1997); however, the major technical hurdle to achieve this therapy for patients is the cheap production of relatively large amounts of the normal enzyme. Genetically modified cells of different origin expressing a high level of enzyme may represent an *in vivo* continuous source of the missing protein. High levels of recombinant human lysosomal enzymes were obtained *in vitro* (Anson *et al.*, 1992; Bielicki *et al.*, 1996) and *in vivo* (Moullier *et al.*, 1995; Salvetti *et al.*, 1995b) by retrovirus-mediated gene transfer in different MPSs. A phase I/phase II gene therapy clinical trial aimed at increasing the enzyme level in Hunter patients affected by the mild form of the disease was proposed (Whitley *et al.*, 1996).

The clinical exploitation of the missing enzyme produced by genetically modified cells in MPSs requires the development of suitable models of delivery. In β-glucuronidase-deficient mice, which develop a disease similar to human MPS type VII (Sly syndrome), it was shown by Moullier *et al.* (1993) that continuous enzyme replacement could be achieved by intraperitoneal implants of genetically modified fibroblasts embedded into collagen lattices (neo-organs). This approach led to a complete disappearance of the typical lysosomal storage lesion in liver and spleen and partially prevented GAG accumulation and excretion. Moreover, a prolonged secretion at therapeutic levels of the same recombinant protein was obtained by the engraftment of genetically engineered myoblasts into the tibialis anterior muscle of the adult immunocompetent β-glucuronidase deficient mouse (Naffakh *et al.*, 1996).

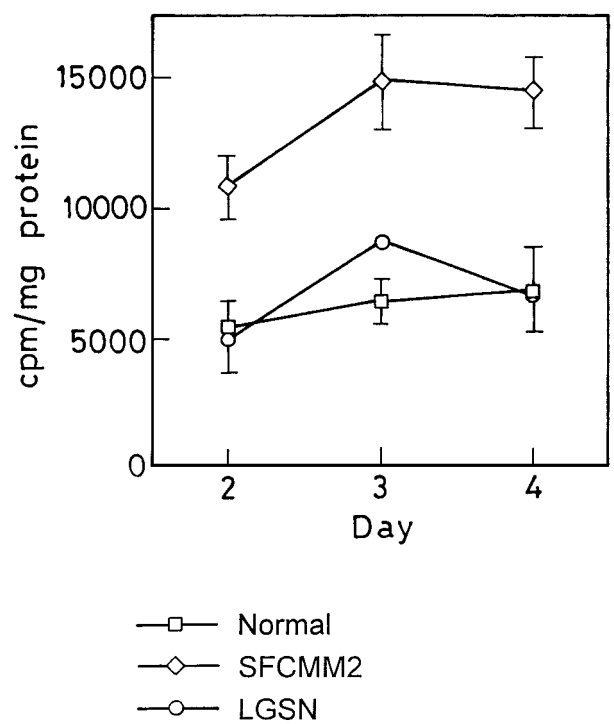

FIG. 3. $^{35}SO_4$GAGs accumulation in LGSN-transduced Morquio fibroblasts. Fibroblasts derived from Morquio patients were transduced either with the LGSN or the SFCMM2 control vectors and selected in G418. Cells were harvested at days 2, 3. and 4 and assayed for $^{35}SO_4$ accumulation and protein content (mean <+/−> SD, $n = 2$) as described in Materials and Methods. (□) Normal fibroblasts; (◇) LGSN-transduced Morquio fibroblasts; (○) SFCMM2-transduced Morquio fibroblasts.

This study was aimed at checking the feasibility of a genetic strategy devised to retard or even stop mucopolysaccharide accumulation in Morquio cells by supplying additional enzyme. Thus, we analyzed the production of GALNS from different transduced cell lines, *i.e.*, fibroblasts, PBLs, keratinocytes, muscle cells, and synoviocytes. Actually, the level of enzyme secretion we obtained by transduced fibroblasts and muscle cells was similar (compared to the normal values) to that reported for the β-glucuronidase enzyme in the two aforementioned studies. This observation, along with the metabolic correction we achieved by transducing Morquio fibroblasts, substantiates the idea that a similar gene therapy approach can be proposed for Morquio disease. In particular, our data support the hypothesis that skin fibroblasts, muscle cells, and epidermal keratinocytes could be used as a source of GALNS, as confirmed by the reduction in GAGs accumulation we achieved in Morquio fibroblasts (see the Results section). In contrast, PBLs may not represent a suitable vehicle to produce supplemental GALNS. Keratinocytes can be serially propagated in culture and then grafted onto the donor to generate epidermis (Green *et al.*, 1979). Cultured keratinocytes can be genetically engineered to synthesize products of exogenous genes and, if the transferred gene encodes a secreted protein, cultured keratinocytes have been shown to secrete both *in vitro* and after grafting (Fenjves *et al.*, 1994). This raises the possibility of using keratinocytes to introduce therapeutic enzyme to the circulation. Moreover, the long-term success of cultured epithelial autografts in treating burn patients suggests that epidermal stem cells are present in culture (Barrandon and Green, 1987). We provide the first evidence that keratinocytes could express high level of active GALNS and therefore could be used as a secreting cell for *in vitro* cross-correction of MPS IVA cells.

A generalized enzyme supplement via peripheral enzyme source will on its own probably not be sufficient to restore efficiently the function of the most severely effected organs. The ideal therapeutic strategy in MPSs therefore would consist of a number of combination of targeted correction of the most severely affected organs and a generalized enzyme supplement, to ensure a minimal homeostatic threshold. Gene therapy has been proposed as an approach for delivery proteins to arthritic joints and is under investigation to verify if it is safe, feasible, and well tolerated in humans and whether it leads to intraarticular expression of transferred gene and evidence of an appropriate biological response (Bandara *et al.*, 1992; Evans *et al.*, 1996). Such an approach may be useful to treat locally a number of other inherited connective tissue diseases including mucopolysaccharidoses (Evans and Robbins, 1995). To mimic an *in vitro* model of an MPS IVA joint, we have co-cultured retrovirus-transduced rabbit synoviocytes with human Morquio fibroblasts and we have assayed an increase of GALNS activity in Morquio cells.

The data collected in this work represent a necessary step before *in vivo* experimentation in the Morquio animal model, which we are developing at the moment after the cloning of the mouse gene (Montano *et al.*, 2000). Nevertheless, these *in vitro* data show that gene therapy for Morquio disease may be practicable. Due to the multiorgan manifestation of the symptoms of the disease, the choice of the cells to be genetically modified as a supplemental source of the missing enzyme remains to be further explored.

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