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# Metabolic correction and cross-correction of mucopolysaccharidosis type II (Hunter syndrome) by retroviral-mediated gene transfer and expression of human iduronate-2-sulfatase

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**ABSTRACT** **To explore the possibility of using gene transfer to provide iduronate-2-sulfatase (IDS; EC 3.1.6.13) enzyme activity for treatment of Hunter syndrome, an amphotropic retroviral vector, L2SN, containing the human IDS coding sequence was constructed and studied for gene expression *in vitro*. Lymphoblastoid cell lines (LCLs) from patients with Hunter syndrome were transduced with L2SN and expressed high levels of IDS enzyme activity, 10- to 70-fold higher than normal human peripheral blood leukocytes or LCLs. Such L2SN-transduced LCLs failed to show accumulation of $^{35}SO_4$ into glycosaminoglycan ($^{35}SO_4$-GAG), indicating that recombinant IDS enzyme participated in GAG metabolism. Coculture of L2SN-transduced LCLs with fibroblasts from patients with Hunter syndrome reduced the accumulation of $^{35}SO_4$-GAG. These results demonstrated retroviral-mediated IDS gene transfer into lymphoid cells and the ability of such cells to provide recombinant enzyme for intercellular metabolic cross-correction.**

Hunter syndrome, or mucopolysaccharidosis (MPS) type II, is an X chromosome-linked recessive inborn error of metabolism resulting from the deficiency of lysosomal iduronate-2-sulfatase (IDS; EC 3.1.6.13) enzyme activity and the consequent systemic accumulation of glycosaminoglycan (GAG) substrates, heparan sulfate and dermatan sulfate (for review, see refs. 1–3). The disorder is clinically manifest as coarse facial features, dysostosis multiplex, joint contractures, hepatosplenomegaly, and obstructive airway disease. In the severe form, children experience progressive mental retardation and die before 15 years of age. Patients with relatively mild forms have normal intellect, but typically succumb to cardiac and respiratory disease in early adulthood.

Proposed therapies for Hunter syndrome have been based on early experiments with fibroblasts from patients with Hunter syndrome that showed that normal catabolism of GAG could be achieved by coculture with fibroblasts from patients with other MPS diseases (4). More recently, similar results have been obtained by coculture with normal lymphocytes or macrophages (5–8). Such studies provided important insights into the posttranslational processing, trafficking, and reuptake of lysosomal enzymes. Early attempts to replace IDS enzyme activity in patients with Hunter syndrome by infusion of normal plasma (9) or leukocytes (10) yielded equivocal results. However, clinical trials of allogeneic bone marrow transplantation have provided evidence of metabolic correction in some tissues (11). Unfortunately, application of bone marrow transplantation is limited by lack of histocompatible donors, a significant mortality rate of 20–50%, and complications such as graft-versus-host disease.

As a means of circumventing problems associated with marrow transplantation, gene transfer targeted at autologous marrow cells has been proposed as an alternative treatment for lysosomal storage diseases (12–15). To explore gene transfer for treatment for Hunter syndrome, we reconstructed the full-length IDS cDNA in a Moloney murine leukemia virus-based vector. Retroviral-mediated IDS gene transfer into lymphoblastoid cell lines (LCLs) produced very high levels of IDS enzyme activity. Furthermore, we observed that expression of recombinant IDS enzyme contributed to GAG metabolism not only in transduced lymphoid cells but also in nontransduced fibroblasts grown in coculture.

## MATERIALS AND METHODS

**DNA Manipulations.** Restriction enzymes were obtained from New England Biolabs. DNA polymerase I (Klenow fragment) and T4 DNA ligase were obtained from Bethesda Research Laboratories. Plasmids were maintained in *Escherichia coli* K-12 294 and harvested by alkaline lysis (16). Sequencing was performed using Sequenase (United States Biochemical) and dideoxynucleotide chain-termination (17). PCR was conducted using *Taq* DNA polymerase (Cetus) in an automated thermal cycler (Perkin–Elmer/Cetus).

**Construction of pL2SN.** For construction of the retrovirus pL2SN (Fig. 1), the 3′ portion of the IDS coding sequence was generated by reverse transcription linked to PCR using total RNA extracted from human leukocytes as template (20). A *Bam*HI site was introduced at base 1669 (enumerated relative to the translational start site) by reamplification of the 3′ product using an antisense oligonucleotide primer IDS2 (5′-CATTTGGGATCCATGGTTGG-3′) and a sense primer IDS3 (5′-ATGAAAACGTCAGCCAGTCC-3′). PCR conditions were 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C for 30 cycles. The 3′ segment of the IDS cDNA (bases 1347–1669) was cloned into the *Bam*HI site of pBluescript II (Stratagene) and recovered as an *Eco*RI–*Bam*HI fragment (bases 1425–1669). The 5′ portion of the IDS coding sequence was excised from pc2S15 (generously provided by J. J. Hopwood, Adelaide Children's Hospital, North Adelaide, Australia) as an *Eco*RI fragment (bases −18 to 1425). The 5′ and 3′ segments of the IDS cDNA were then inserted in a three-way ligation between the *Eco*RI and *Bam*HI sites of the retroviral vector pLXSN (21). Correct assembly of the retroviral vector, including orientation of the 5′ region of IDS in pL2SN, was

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Abbreviations: GAG, glycosaminoglycan; IDS, iduronate-2-sulfatase; LCL, lymphoblastoid cell line; MPS, mucopolysaccharidosis; U, unit(s); LTR, long terminal repeat.

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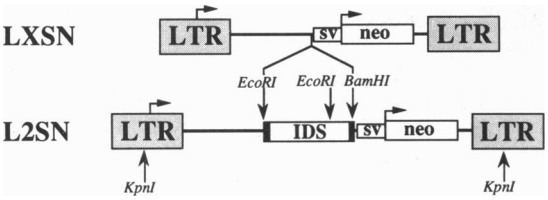


FIG. 1. Schematic diagram of retrovirus construct L2SN. The vector was assembled by reconstruction of the IDS coding sequence in the Moloney murine leukemia virus-derived vector LXSN (18). The complete IDS coding sequence was obtained by combining a 1.4-kb *Eco*RI–*Eco*RI segment from pc2S15 (19) with the 3'-end segment derived in this laboratory by reverse transcription–PCR. For construction of L2SN, six clones containing the 3'-end *Bam*HI–*Bam*HI fragment were isolated and sequenced. One had the silent mutation $G^{1660} \rightarrow A$ (presumably resulting from *Taq* polymerase infidelity of during PCR amplification) and was incorporated into L2SN as an identifying marker. The sequence of the IDS coding region was otherwise identical to the published coding sequence (19). The final L2SN construct contained the IDS cDNA transcriptionally regulated by the long terminal repeat (LTR) and the selectable marker gene for neomycin phosphotransferase (neo) regulated by the simian virus 40 early promoter. Other sequences are Moloney murine leukemia virus and Moloney murine sarcoma virus LTRs and simian virus 40 early promoter (SV). The locations of *Bam*HI and *Eco*RI sites used for construction of the IDS sequence and the *Kpn* I sites used for Southern blot analysis (Fig. 2) are indicated.

confirmed by restriction mapping and sequence analysis of the entire IDS coding region.

**Mammalian Cell Culture and Retroviruses.** Epstein–Barr virus-transformed LCLs established from peripheral blood lymphocytes of normal individuals ($LCL_{Normal}$) and patients with Hunter syndrome ($LCL_{MPS}$) were obtained from the Immunobiology Research Center, University of Minnesota. Specific mutations of IDS-deficient LCLs were identified as described (20, 22), one cell line having a complete deletion of the coding region ($LCL_{MPSdel}$) and the other a point mutation $C^{1530} \rightarrow A$ ($LCL_{MPSP469H}$). LCLs were grown in plastic flasks (T-75, Falcon, Becton Dickinson) at 0.5–2 × $10^6$ cells per ml in RPMI 1640 medium (GIBCO) with 15% (vol/vol) heat-inactivated fetal calf serum (HyClone). Cultured human fibroblasts were grown in plastic dishes (3003, Falcon) in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% heat-inactivated fetal calf serum. NIH 3T3 cells and murine retroviral-packaging cell lines GP+E86 (23) and PA317 (18) were grown in DMEM with 10% heat-inactivated newborn calf serum (HyClone). All media were supplemented with 2 mM glutamine (Sigma), penicillin (50 units/ml), streptomycin sulfate (50 mg/ml), and Fungizone (125 mg/ml) (GIBCO). Penicillin, streptomycin sulfate, and Fungizone were omitted for experiments employing $^{35}SO_4$ labeling of GAG (below). Cultures were incubated in a humidified atmosphere of 5% $CO_2$/95% air at 37°C.

Retrovirus constructs were packaged by transfection into GP+E86 cells using DNA–calcium phosphate coprecipitation (24, 25). Transiently generated virus was then used to transduce PA317 cells (21) isolating G418-resistant (0.4 mg of active base per ml, GIBCO/BRL) clones and screening for those producing the highest titer of G418-resistant virus using NIH 3T3 cells as targets (26).

**Transduction and Selection.** LCLs were transduced by incubating overnight for 3 consecutive days with retroviral supernatant (2:1 colony-forming units/cell ratio) and Polybrene (8 μg/ml, Sigma). LCLs were expanded for 2 weeks. To select for transductants, LCL cultures were then grown in medium containing G418 (0.4 mg/ml) for 2 additional weeks and, thereafter, maintained in medium containing G418 (1.0 mg/ml).

**Southern Blot Analysis.** To verify transduction and integrity of proviral integrants by Southern blot analysis, genomic DNA was isolated, digested with *Kpn* I, and electrophoresed in 0.8% agarose gel prior to transfer to Zetabind membrane (Cuno). The membrane was baked at 80°C for 2 hr and then washed twice in 0.1× standard saline citrate (SSC)/0.1% SDS at 60°C for 1 hr. Prehybridization was in 5.4× standard saline/phosphate/EDTA (16)/1500 units (U) of heparin/11× Denhardt's solution/0.8% SDS/salmon sperm DNA (200 μg/ml)/50% (vol/vol) formamide for 6 hr at 42°C. To probe for IDS sequences, the *Eco*RI fragment of pc2S15 was purified and $^{32}P$-radiolabeled by random priming (27). After overnight hybridization in prehybridization solution and IDS probe, the membrane was washed three times in 0.1× SSC/0.1% SDS for 15 min at ambient temperature and three times in 0.1× SSC/0.1% SDS for 30 min at 60°C.

**Determination of Enzyme Activities.** Peripheral blood leukocytes and LCLs were disrupted by one freeze–thaw cycle and then sonicated (three 15-s bursts at level 7, Branson Sonifier 450) in 1% Triton X-100. Cell sonicates, or culture media, were then desalted into distilled water by centrifugal column chromatography (Bio-Gel P-10, fine mesh, Bio-Rad). IDS enzyme activity was measured in extracts using the tritiated disaccharide substrate L-*O*-(α-iduronate-2-sulfate)-(1 → 4)-D-*O*-2,5-anhydro[1-$^3$H]mannitol 6-sulfate (Research and Development Limited Partnership, Toronto) as described (28, 29). Protein concentration was measured with Coomassie blue (Bio-Rad) by the method of Bradford (30). One unit of enzyme activity was defined as 1% of total $^3H$ substrate converted to product.

Lysosomal glycosidase activities were measured using the appropriate fluorogenic 4-methylumbelliferyl-glycoside substrates and expressed as nmol per mg of protein per hr for cells or as nmol per ml of medium per hr for culture medium.

**$^{35}SO_4$-GAG Labeling and Assay.** For studies assessing GAG metabolism, cultured cells (i.e., LCLs, fibroblasts, etc.) were grown for 2 days in minimal essential medium [22300, GIBCO, $NaHCO_3$ (pH 6.9) at 1.6 g/liter] with $CaCl_2$ (140 mg/liter)/15% fetal calf serum/2 mM glutamine. Cellular GAG was metabolically labeled by addition of $^{35}SO_4$ (Amersham) to culture medium (4 μCi/ml; 1 Ci = 37 GBq). To study GAG clearance, fibroblasts were labeled 2 days in $^{35}SO_4$ and then subcultured by trypsinization into 30-$cm^2$ plates (3002, Falcon). After cocultivation with LCLs for 2 additional days, adherent fibroblast monolayers were washed twice with phosphate-buffered saline, harvested by trypsinization, collected, lysed with 0.1 ml of 0.5 M NaOH, and then neutralized with 0.05 ml of 1 M HCl. Macromolecular material was separated from unincorporated $^{35}SO_4$ by centrifugal column chromatography.

## RESULTS

**Construction and Packaging of Retroviral Vector L2SN.** To assess the potential of gene transfer for correcting the metabolic defect in cells from patients with Hunter syndrome, we constructed a recombinant retroviral vector, pL2SN (Fig. 1). L2SN was packaged by shuttling into PA317 cells, thus establishing an amphotropic virus-producer cell line that generated an overnight supernatant with a titer of 2 × $10^6$ G418-resistant colony-forming units/ml.

**L2SN Transduction of Lymphoblastoid Cell Lines from Patients with Hunter Syndrome.** Epstein–Barr virus-transformed LCLs from patients with Hunter syndrome ($LCL_{MPS}$) and normal individuals ($LCL_{Normal}$) were transduced with the L2SN vector or the control vector LXSN (Fig. 1). Heterogeneous populations of transductants were selected in medium containing G418. Presence of the full-length provirus L2SN in $LCL_{MPS}$-L2SN was verified by PCR (data not shown). Further confirmation of intact L2SN trans-

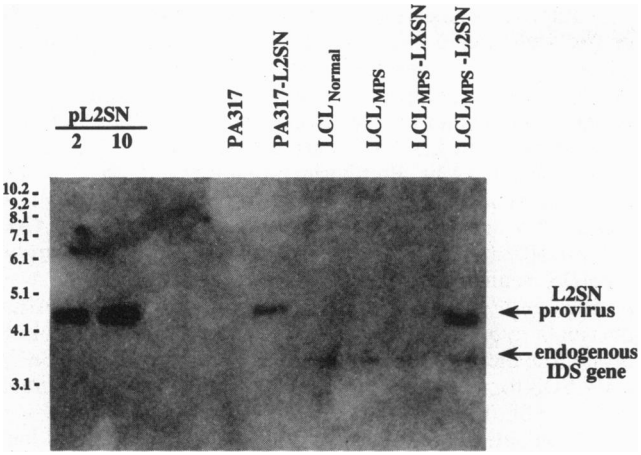


FIG. 2. Southern blot analysis. Genomic DNA was digested with *Kpn* I, blotted, and probed with the partial IDS cDNA clone pc2S15. Lanes contained DNA (as indicated, from left to right) from the following sources: 2 and 10 pg of pL2SN, murine PA317 packaging cell line, PA317-L2SN virus-producer cell line, $LCL_{Normal}$-LXSN transduced cell line, $LCL_{MPSP469H}$ ($LCL_{MPS}$) cell line (untransduced), transduced cell line $LCL_{MPSP469H}$-LXSN after selection with G418, and transduced cell line $LCL_{MPSP469H}$-L2SN after G418 selection. Migration of molecular size standards is indicated on the left (in kb), and locations of the L2SN provirus and the endogenous IDS gene are indicated on the right.

duction and proviral integration in PA317 and $LCL_{MPS}$ was obtained by Southern blot analysis (Fig. 2). Probing *Kpn* I-digested genomic DNA from these cell lines with an IDS-specific fragment from pc2S15 (19) revealed a 4.3-kb proviral band that comigrated with *Kpn* I-digested plasmid pL2SN. No cross-hybridization with the endogenous murine IDS gene was observed in samples from the packaging cell lines.

**IDS Enzyme Activity in Normal and Transduced Lymphoid Cells.** IDS enzyme activity was assayed in sonicates of $LCL_{MPS}$-L2SN, control-transduced, and normal cell populations (Table 1). IDS was not detectable (<10 U per mg per hr) in $LCL_{MPS}$ or in $LCL_{MPS}$-LXSN cells but $LCL_{Normal}$ cells contained readily measurable IDS enzyme activity (mean, 829 U per mg per hr). In contrast, $LCL_{MPSP469H}$-L2SN cells contained substantially increased levels of IDS activity (mean, 8770 U per mg per hr), levels ≈10-fold greater than $LCL_{Normal}$ or normal leukocytes (mean, 807 U per mg per hr). Another transduced cell line, $LCL_{MPSdel}$-L2SN, contained even higher levels of IDS activity (mean, 55,800 U per mg per hr), ≈70-fold greater than $LCL_{Normal}$. The highest levels were obtained after 2 months of G418 selection.

Table 1. IDS enzyme activity in leukocytes and cultured LCLs

| Cell type | IDS enzyme activity, U per mg of protein per hr | |
|---|---|---|
| | Mean ± SD (*n*) | Range |
| WBC, uncultured | | |
| $WBC_{Normal}$ | 807 ± 252 (23) | 418–1250 |
| $WBC_{MPS}$ | <10 (3) | |
| Untransduced LCL* | | |
| $LCL_{Normal}$ | 829 ± 131 (8) | 641–970 |
| $LCL_{MPS}$ | <10 (7) | |
| Transduced LCL† | | |
| $LCL_{MPSP469H}$-LXSN | <10 (10) | |
| $LCL_{MPSP469H}$-L2SN | 8770 ± 3400 (10) | 3100–12,300 |
| $LCL_{MPSdel}$-LXSN | <10 (5) | |
| $LCL_{MPSdel}$-L2SN | 55,700 ± 18,700 (5) | 27,700–76,500 |

WBC, leukocyte.
*Cultured without G418.
†Selected for LXSN-transduced or L2SN-transduced cells by culture with G418.

**Lysosomal Enzyme Release.** To determine the effect of retroviral transduction and overexpression of recombinant IDS on lysosomal enzyme release, transduced cells were cultured for 2 days in fresh medium and then studied for several lysosomal enzyme activities. Cellular IDS activity in $LCL_{MPSdel}$-L2SN was relatively high (mean, 58,200 U per mg per hr), ≈120-fold greater than control $LCL_{Normal}$-LXSN levels (mean, 489 U per mg per hr). The levels of IDS activity in medium that had not been exposed to cells (mean, 1.72 U per ml per hr) or levels in medium harvested from $LCL_{MPS}$-LXSN (3.31 U per ml per hr) were relatively low in comparison to levels in medium after culture with $LCL_{Normal}$-LXSN (10.1 U per ml per hr). IDS enzyme activity in medium from $LCL_{MPS}$-L2SN was 46.0 U per ml per hr, ≈5-fold higher than that of $LCL_{Normal}$-LXSN cells.

In $LCL_{MPS}$-L2SN cells, α-mannosidase (197 ± 40.9 nmol per mg per hr; mean ± SD) was significantly reduced compared to $LCL_{MPS}$-LXSN (301 ± 63.7 nmol per mg per hr; matched pairs $P = 0.025$). Similarly, α-fucosidase in $LCL_{MPS}$-L2SN (85.0 ± 14.1 nmol per mg per hr) was reduced compared to levels in $LCL_{MPS}$-LXSN (157 ± 31.3 nmol per mg per hr; $P = 0.029$). However, other lysosomal glycosidase activities (β-mannosidase, total α-galactosidase, β-galactosidase, β-hexosaminidase A, and β-glucuronidase) were not significantly altered.

In the medium of $LCL_{MPS}$-L2SN cells that were overexpressing IDS enzyme, none of the glycosidase activities were significantly different from levels in the medium of either $LCL_{Normal}$-LXSN or $LCL_{MPS}$-LXSN control cultures.

**$^{35}SO_4$-GAG Accumulation in LCLs.** To evaluate the ability of L2SN transduction to correct the metabolic defect, $^{35}SO_4$-GAG levels were studied in LCL cultures (Fig. 3). After initial incorporation of $^{35}SO_4$ during the first 24 hr, no further accumulation was observed in $LCL_{Normal}$-LXSN. In contrast, IDS-deficient $LCL_{MPS}$-LXSN showed continued accumulation of $^{35}SO_4$-GAG through 3 days in culture. However, when $LCL_{MPS}$ was transduced with L2SN, a steady state of $^{35}SO_4$ metabolism was observed similar to $LCL_{Normal}$-LXSN with no accumulation after the initial day of incorporation. Thus, transduction by L2SN not only produced high levels of IDS activity but also corrected aberrant GAG metabolism in L2SN-transduced $LCL_{MPS}$ cells.

**$^{35}SO_4$-GAG Clearance: Metabolic Cross-Correction.** To evaluate the ability of $LCL_{MPS}$-L2SN to effect metabolic

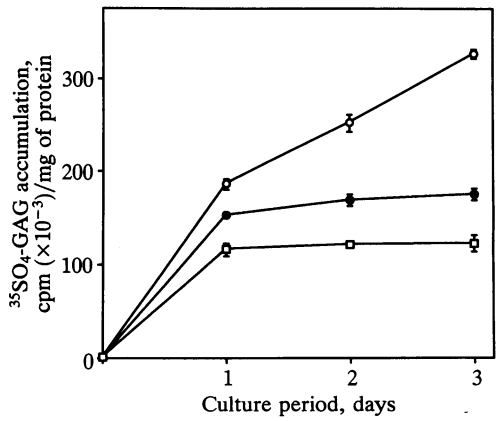


FIG. 3. $^{35}SO_4$-GAG accumulation by transduced LCLs. Transduced LCLs were grown in sulfate-free medium supplemented with $^{35}SO_4$. After growth for the specified interval, cells were washed and assayed for macromolecular $^{35}S$. Cellular $^{35}SO_4$-GAG incorporation was expressed as cpm/mg of protein. Data are the mean ± SD; $n$ = 3 for cell lines $LCL_{MPSP469H}$-LXSN (○), $LCL_{MPSP469H}$-L2SN (●), and $LCL_{Normal}$-LXSN (□).

correction in neighboring nontransduced cells (i.e., cross-correction), enzyme-deficient fibroblasts from a patient with Hunter syndrome were grown in $^{35}SO_4$ for 2 days to pulse-label a pool of $^{35}SO_4$-GAG. Labeled fibroblasts were then cocultured with either $LCL_{MPS}$-LXSN or $LCL_{MPS}$-L2SN in $^{35}SO_4$-free medium to assess the rate of clearance of accumulated $^{35}SO_4$-GAG. Fibroblasts cocultured for 2 days with various doses of $LCL_{MPS}$-LXSN (0.5, 1.0, 2.5, or 4.0 × $10^6$ cells per ml) showed some reduction in $^{35}SO_4$-GAG (to 46, 31, 57, and 45% of initial cpm, respectively). In contrast, coculture with $LCL_{MPS}$-L2SN resulted in significant (matched pairs *t* test, $P = 0.019$) and nearly complete clearance of $^{35}SO_4$-GAG (to 16, 15, 5, and 7%, respectively). These results suggested that recombinant IDS was expressed in a form that was exported from L2SN-transduced $LCL_{MPS}$ and, subsequently, taken up by cocultured fibroblasts.

**Comparison of Cross-Correction by $LCL_{Normal}$–LXSN and $LCL_{MPS}$–L2SN.** Because L2SN-transduced $LCL_{MPS}$ had much higher levels of IDS enzyme activity than $LCL_{Normal}$, we predicted that $LCL_{MPS}$-L2SN might also be more effective at accomplishing cross-correction. To test this hypothesis, fibroblasts from patients with Hunter syndrome were cultured with $^{35}SO_4$ under four conditions. Fibroblasts were cultured alone or cocultured with one of the three transduced cell lines: $LCL_{MPS}$-LXSN, $LCL_{Normal}$-LXSN, or $LCL_{MPS}$-L2SN. After metabolic labeling for 2 days, adherent fibroblasts were washed of medium and nonadherent LCLs and then analyzed for $^{35}SO_4$-GAG content. As shown in Fig. 4, accumulation of $^{35}SO_4$-GAG after coculture with $LCL_{MPS}$-LXSN was not significantly different from that of fibroblasts cultured alone ($P = 0.42$). However, fibroblasts cocultured with $LCL_{Normal}$-LXSN showed decreased $^{35}SO_4$ accumulation (mean, 68% of fibroblasts cultured alone), which was significantly different ($P = 0.04$) from fibroblasts cocultured with $LCL_{MPS}$-LXSN. Fibroblasts cocultured with $LCL_{MPS}$-L2SN demonstrated even lower $^{35}SO_4$-GAG accumulation (58%), a reduction that was significantly different ($P < 0.01$) from fibroblasts cocultured with $LCL_{MPS}$-LXSN. This greater metabolic cross-correction by $LCL_{MPS}$-L2SN was presumably the result of increased retroviral IDS enzyme expression, release, and uptake by fibroblasts.

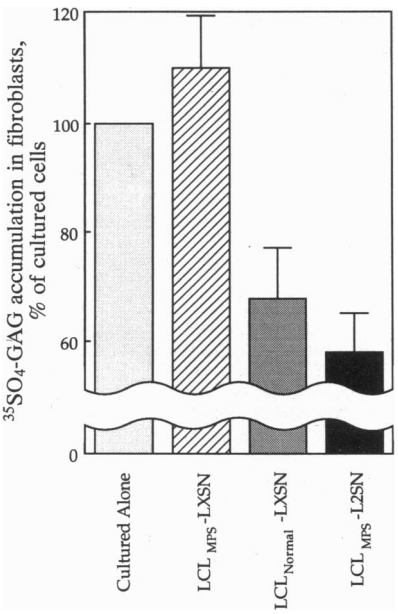


FIG. 4. Accumulation of $^{35}SO_4$-GAG by enzyme-deficient fibroblasts cultured alone or cocultured with transduced $LCL_{Normal}$ or $LCL_{MPSP469H}$ ($LCL_{MPS}$). IDS-deficient fibroblasts from patients with Hunter syndrome were cultured alone or cocultured with retrovirus-transduced LCLs (3 ml of 0.8 × $10^6$ LCLs per ml in 9.6-$cm^2$ plates) for 2 days in medium containing $^{35}SO_4$. Fibroblasts were then washed, extracted, and assayed for macromolecular $^{35}S$. The $^{35}SO_4$-GAG remaining in fibroblasts cocultured with transduced LCLs is expressed as a percent of that remaining in fibroblasts cultured alone (mean ± SEM; $n = 3$ fibroblast cell lines derived from different patients with Hunter syndrome).

## DISCUSSION

We constructed a recombinant retroviral vector L2SN to mediate insertion and expression of human IDS cDNA in lymphohematopoietic target cell populations. As a model system for studying gene transfer, expression of enzyme and metabolic correction, LCL established from patients with Hunter syndrome were targeted for L2SN transduction. Cells transduced with L2SN were selected in medium containing G418 and were found to contain a remarkably high level of IDS enzyme activity, levels 10- to 70-fold greater than either normal LCLs or normal human leukocytes. Recombinant IDS enzyme appears to undergo essentially normal posttranslational modification and trafficking to lysosomes as indicated by a substantial reversal of GAG substrate accumulation, not only in L2SN-transduced $LCL_{MPS}$ but also in cocultivated Hunter fibroblasts.

We propose that this high level of IDS expression mediated by the strong heterologous promoter is characteristic of lysosomal enzymes in general. Several recent studies demonstrating expression of cDNA for other human lysosomal enzymes have observed similarly high levels of expression from heterologous promoters. Arylsulfatase A was expressed in fibroblasts at levels 10-fold greater than that of normal cells (31), acid sphingomyelinase was expressed 16-fold above normal (32), arylsulfatase B was overexpressed by 36-fold (33), α-L-iduronidase was expressed at 50- to 250-fold (12), and α-L-fucosidase was expressed 30- to 400-fold over normal levels (34). Our results with IDS are thus consistent with the high levels of lysosomal enzyme expression after gene transfer when the coding sequence is transcriptionally regulated by strong heterologous promoters. Conversely, this implies a relatively low level of basal expression by endogenous lysosomal enzyme genes in the native state.

As early as 1963, Bernlohr (35) suggested that expression of lysosomal enzymes at normal levels is well in excess of the metabolic demand and that altered kinetics of an aberrant enzyme would lead to a higher steady-state concentration of substrate or even progressive accumulation in affected individuals. More recently, Sandhoff and colleagues expanded upon this kinetic model proposing a relationship between the phenotype (e.g., age of onset and clinical severity) and small quantitative differences in residual enzyme activity (36); they subsequently provided experimental data correlating the residual activity of lysosomal enzymes and the turnover of substrate in cell culture (37). This model implies that only a small increment in catalytic activity may be sufficient to prevent progressive substrate accumulation and achieve a steady state of substrate metabolism. These observations thus suggest that overexpression of the appropriate cDNA by a small number of transduced cells, with increased release and transfer, may provide sufficient therapeutic enzyme to normalize lysosomal metabolism systemically in genetically deficient patients. This prediction has been borne out by stem-cell gene transfer studies in the MPS VII murine model for which a very small amount of enzyme reversed pathology (15).

The phenomenon of lysosomal enzyme transfer and normalization of substrate accumulation was first observed in experiments studying genetic complementation by cocultivation of fibroblasts from patients with Hunter syndrome and other MPS diseases (4). Such studies have provided the rationale for enzyme replacement therapy. The success of

allogeneic bone marrow transplant to provide a source of enzyme that results in clearance of GAG from tissues in humans (38–41) and in animals (42, 43) has fueled speculation that gene transfer into autologous lymphohematopoietic cells may provide an alternate therapy.

Recent *in vitro* studies of retrovirus-mediated gene expression have shown that several recombinant lysosomal enzymes become localized to the lysosome of transduced cells (12, 31) and normalize substrate degradation (12, 15, 31), suggesting that posttranslational modification and trafficking of recombinant enzyme is normal. Other studies of purified recombinant lysosomal enzymes have suggested that these enzymes are properly glycosylated for uptake (12, 44, 45). However, extreme overexpression may result in intracellular aggregation of recombinant enzyme (45) or interference with trafficking of other lysosomal enzymes and optimal substrate catabolism (12). To our knowledge, it was not previously known whether transduced cells were capable of direct enzyme release and reuptake by nontransduced cells. Here we observed that L2SN transduction corrected GAG metabolism in retrovirus-transduced $LCL_{MPS}$ and, importantly, also in nontransduced fibroblasts grown in coculture. Thus, transduced cells are shown to provide a direct source of transferable recombinant enzyme capable of mediating intercellular cross-correction. Notably, we observed increased release of IDS into the culture medium; we did not observe the generalized pattern of lysosomal enzyme release that occurred with overexpression of α-L-iduronidase (12) but was not observed with overexpression of α-galactosidase (45).

The very high level of IDS activity expressed by retroviral-mediated gene transfer in LCLs furthers the argument that genetic manipulation of autologous cells may provide greater quantities of enzyme than is supplied by bone marrow transplantation. Our results extend this observation also demonstrating that L2SN-transduced LCLs have an increased ability to mediate cross-correction. Importantly, this *in vitro* model of gene therapy using LCLs as the target for retrovirus transduction parallels a currently feasible maneuver exploiting IDS expression in lymphocytes, the therapeutic system currently being evaluated in clinical trials of lymphocyte gene therapy for adenosine deaminase deficiency (46, 47). Experimentally, lymphoid cells have been shown to mediate lysosomal enzyme transfer to cultured fibroblasts by mechanisms involving cell–cell contact (8) and may also contribute enzyme for uptake by mannose-6-phosphate receptors in patients treated by allogeneic bone marrow transplant (48). The use of genetically manipulated autologous cells as a source of enzyme may be appropriate for patients with mild Hunter syndrome facing the life-threatening complications of obstructive airway disease but who lack a suitable donor for marrow transplant. However, the most therapeutically challenging manifestation of Hunter syndrome is the neurological disease of severely affected patients; it is currently unclear whether recombinant IDS enzyme expressed in lymphocytes would enter the central nervous system.

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