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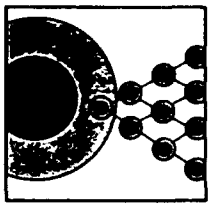

Rapid Communication

Correction of the enzyme deficiency in hematopoietic cells of Gaucher patients using a clinically acceptable retroviral supernatant transduction protocol*

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Abstract

Gaucher disease is a lysosomal storage disorder caused by a deficiency of the enzyme glucocerebrosidase (GC), and is an excellent candidate for gene replacement therapy. To develop a clinically acceptable protocol for this purpose, we created two amplified (A) high-titer retroviral vector–producer cell lines to efficiently transduce hematopoietic stem and progenitor cells. GP+envAm12/A-LGSN (A-LGSN), contained the GC cDNA driven by the retroviral long terminal repeat (LTR) and the neomycin phosphotransferase gene expressed from the simian virus 40 early promoter. GP+envAm12/A-LG4 (A-LG4) contained only the GC gene driven by the LTR. Both A-LGSN and A-LG4 contained multiple proviral copies and gave approximately 10-fold higher titers on 3T3 cells compared to their unamplified counterparts. These vectors were packaged in GP+envAm12 cells because vectors produced in this cell line transduced hematopoietic cells more efficiently than other packaging cells tested. Bone marrow mononuclear cells and purified $CD34^+$ cells were infected with virus supernatants four times in the presence of interleukin-3 (IL-3), IL-6, and stem cell factor (SCF) over 96 hours in culture. Cells were then plated in semisolid cultures and colony-forming unit–granulocyte/macrophage (CFU-GM) colonies were scored for vector presence by polymerase chain reaction (PCR). Transduction efficiency of CFU-GM colonies derived from $CD34^+$ cells was improved considerably using the amplified vectors in the GP+envAm12 packaging line. For A-LGSN, A-LG4, and unamplified LGSN, transduction efficiencies were 41, 42, and 25%, respectively. Therefore, multiple proviral copies resulting in higher titer improves retroviral transduction of human hematopoietic progenitor cells. Hematopoietic cells from Gaucher patients were transduced and placed into long-term bone marrow culture (LTBMC). Viral supernatant from the amplified producer lines transduced long-term culture initiating cells (LTCIC) efficiently (30 to 50%) using this clinically acceptable protocol. Both sustained mRNA expression and GC enzyme production are achieved in the long-term culture of LTCIC and lead to correction of the GC deficiency in their progeny cells.

Key words: GC deficiency—Gene therapy—Gaucher disease—Retroviral supernatant—Transduction

Introduction

Gaucher disease is an autosomal recessive lysosomal storage disorder characterized by a deficiency of GC and an accumulation of glucocerebroside in bone marrow–derived macrophages [1]. A variety of clinical manifestations of this disease have been described, including hepatosplenomegaly, hypersplenism, pancytopenia, and bone deterioration [2]. Recently, enzyme replacement therapy [3–5] and allogeneic bone marrow transplantation [6,7] have offered promising therapeutic alternatives. However, enzyme replacement is an expensive intravenous therapy that must be continued throughout the patient's lifetime, and allogeneic bone marrow transplantation is associated with severe morbidity and mortality and cannot be applied universally [8]. Therefore, development of alternative therapeutic approaches is desirable.

Somatic cell gene therapy, delivery of a therapeutic gene into an abnormal cell, has already been used successfully in the treatment of ADA deficiency, and numerous clinical trials are underway [9–11]. In studies for Gaucher disease, the human GC gene has been transferred into hematopoietic progenitor cells [12,13] and hematopoietic stem cells (HSC) of mice [14–17]. Expression of potentially therapeutic levels of human GC enzyme was found in the majority of macrophages from long-term reconstituted mice transplanted with retrovirally transduced HSC [17,18]. In addition, retroviral-mediated transfer of normal human GC cDNA into CFU-GM hematopoietic progenitor cells from Gaucher patients has been demonstrated in a transient culture system; however, the enzyme deficiency could be corrected only after selection of the transduced cells due to poor expression of the transferred GC gene [19]. Most recently, Nolta et al. [20] employed a coculture transduction protocol to transfer the GC gene into more primitive hematopoietic progenitor cells of a Gaucher patient and demonstrated physiologically relevant enzyme expression in progeny cells 1 month after the transduction.

Coculture infection procedure was previously used for transfer of potentially therapeutic genes into human

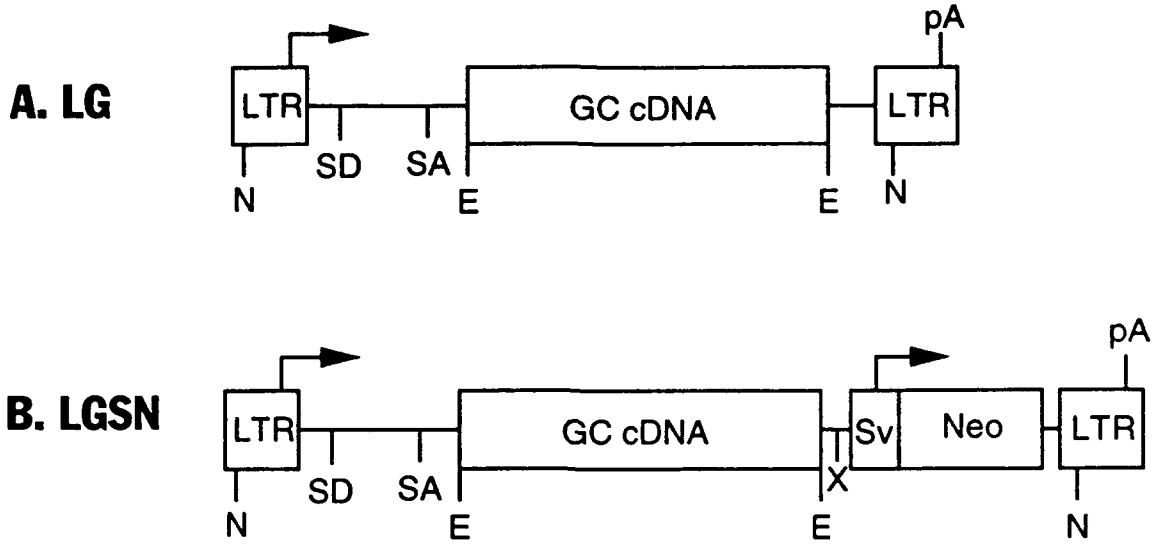

Fig. 1. Retroviral vectors used for GC cDNA transfer. **A.** LG, a murine retrovirus-based vector that includes the GC cDNA driven by the MoMLV LTR. **B.** LGSN, a vector containing the GC cDNA driven by the LTR and the neomycin phosphotransferase gene (Neo) expressed from the simian virus 40 early promoter (Sv). SD = splicing donor; SA = splicing acceptor; pA = polyadenylation site; E = *EcoRI*; N = *Nhe* I; X = *Xho* I. Arrows indicate transcriptional start sites and direction of transcription.

hematopoietic cells because it was difficult to achieve efficient transduction of human hematopoietic cells by supernatant infection techniques. An innovative approach to improve transduction efficiency was presented by Moore et al. [21] using supernatant infection in the presence of stromal cells, but this can be cumbersome in a clinical setting. To develop a clinically acceptable protocol for efficient transfer of a therapeutic gene into primitive hematopoietic progenitor cells, we used two amplified retroviral producer cells (A-LG4 and A-LGSN) containing multiple vector copies to generate high-titer virus supernatant. Transduction efficiency of $CD34^+$ bone marrow cells was improved considerably using supernatants from the amplified producer cell lines, demonstrating that multiple viral copies in producer cells increase their ability to transduce human hematopoietic progenitors. These amplified supernatants were also tested for transduction of mononuclear bone marrow cells and $CD34^+$ bone marrow cells from Gaucher patients. Correction of enzyme activity was seen in the colonies 7 weeks (5 weeks in LTBMC and 2 weeks in CFU-GM assay) after the initial gene transfer procedure. We conclude that LTCIC from patients with Gaucher disease have been efficiently transduced and that correction of the GC enzyme deficiency in their progeny cells has been accomplished.

Materials and methods

Cell culture and retroviral vectors

Psi-CRIP and GP+envAm12 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) (DPBS; Biofluids, Rockville, MD) supplemented with 10% newborn calf serum (NCS-DM) (fetal bovine serum [FBS-DM] was used for PA317 cells), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were passaged every third day after dislodgement with 0.05% trypsin/0.02% EDTA.

Retroviral vectors used in this study, LG and LGSN, were described previously by Correll et al. [18] and Freas et al. [22], respectively. Briefly, LGSN is a murine retrovirus-based vector that includes GC cDNA driven by the LTR and the neomycin phosphotransferase gene expressed from the simian virus 40 early promoter, and LG contains only GC driven by the LTR (Fig. 1).

Virus production

Superinfection of packaging cells was performed to obtain high-titer amphotropic vectors either by coculture [23,24] or by repeated supernatant infections. Amplified GP+envAm12/LGSN clones were obtained by coculturing amphotropic GP+envAm12/LGSN and ecotropic GP+E-86/LGSN for 12 days, during which period kinetics of virus production was measured to determine the optimal length of coculture. Subsequent determination of virus titer demonstrated that coculture of GP+envAm12/LGSN and GP+E-86/LGSN for 6 to 8 days could maximize virus production (Dave et al., unpublished observation).

To generate a high-titer LG vector, superinfection of GP+envAm12 cells was done by repeated application of ecotropic supernatant. Briefly, high-titer viral supernatant from GP+E-86 LG was collected and frozen at –80°C in 3- to 5-mL aliquots. GP+envAm12 packaging cells were split 1:10 and infected the following day with 3 mL GP+E-86/LG supernatant, 7 mL culture medium, and 8 µg/mL polybrene. Infection was repeated daily until cells became confluent. Confluent cells were split again and infected and the procedure was repeated until 12 infections were completed. Individual clones were obtained by plating 100 to 1000 cells on 10-cm culture dishes, and a total of 20 to 40 colonies were isolated. Clone 4 of A-LG (A-LG4) was used in the experiments. Viral titer was determined by infecting $3T3^{tk-}$ cells with 5 mL viral supernatant, and copy number of the provirus in these cells was determined by Southern blot analysis. Helper virus was detected by a marker rescue assay [25,26] using $3T3^{tk-}$ cells containing one copy of the LGSN vector. These cells were infected by the test supernatant, the cells were passaged for 2 weeks, and at that time their supernatant was tested for passage of virus on virgin $3T3^{tk-}$ cells.

Ficoll-gradient separation of mononuclear BM (MNBM) cells

Both normal and Gaucher type I bone marrows were voluntarily donated and harvested by needle aspiration from the posterior iliac crest after informed consent. Bone marrow was diluted 1:3 with 2% FBS-Iscove's medium (FBS-IM), and MNBM cells were obtained by Ficoll-gradient centrifugation at 400*g* at room temperature for 30 minutes on LSM lymphocyte separation medium (Organon Teknika-Cappel, Durham, NC). MNBM cells were washed twice using 2% FBS-IM followed by centrifugation at 1500 rpm for 10 minutes (Sorvall RT6000B). The cell pellet was resuspended either in 2% FBS-IM containing 1000 U/mL DNase I for isolation of $CD34^+$ cells or in LTBMC medium (HCC-5100 myeloid long-term culture medium; Terry Fox Laboratory, Vancouver, Canada) for retroviral infection.

Isolation of BM $CD34^+$ cells

$CD34^+$ cells from MNBM of normal donors and Gaucher patients were purified by positive immunoselection using the CD34 monoclonal antibody KG.1 linked to magnetic Dynabeads (Dynalinc, Fort Lee, NJ) as described elsewhere [27].

BM cell infection and LTBMC

Supernatant infection of hematopoietic BM cells was performed as described by Hughes et al. [28] with modifications. MNBM and BM $CD34^+$ cells were infected at 37°C using fresh virus supernatant supplemented with 2 mM L-glutamine, 8 μg/mL polybrene or 4 μg/mL protamine sulfate, 20 ng/mL IL-3, 50 ng/mL IL-6, and 100 ng/mL SCF at 10^6 cells/mL and 2×10^5 cells/mL, respectively. Approximately 24 hours later, cells were centrifugated at 1000 rpm for 10 minutes and infected under the same conditions as above. The infection procedure was performed once daily for 4 consecutive days. At the end of infection, cells were sampled to determine infection efficiency, and the remaining cells were transferred into T25 ventilated flasks (Costar, Cambridge, MA) at initial densities of 10^6 cells/mL and 2×10^5 cells/mL for MNBM and BM $CD34^-$ cells, respectively, on irradiated feeder layers prepared from heterogenous BM [29] for LTBMC. Cells were maintained at 33°C for 5 weeks, during which time weekly 60% medium change was performed; cell samples were taken for clonogenic assays for CFU-GM and GC enzymatic assays at weeks 3 and 5.

Clonogenic assays for CFU-GM

MNBM and BM $CD34^+$ cells from postinfection cultures or LTBMC were assayed for clonogenic activity by plating 1 to 5×10^4 MNBM cells or 500 BM $CD34^+$ cells in 1 mL HCC-4200's Iscove's methylcellulose (Terry Fox) supplemented with 2 mM L-glutamine and 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) in a 35-mm culture dish to yield 20 to 100 colonies empirically. When cells were taken from late stages of LTBMC, the number of cells plated was doubled. The culture was maintained at 33°C for 2 weeks. Well-isolated colonies were picked for detection of GC cDNA by PCR analysis, and the remainder of the colonies were pooled for GC enzyme assay.

Detection of GC cDNA in CFU-GM by PCR

Individual CFU-GM colonies were picked and suspended in cold DPBS and microcentrifugated for 20 to 30 seconds. Pellets were resuspended in 10 to 20 μL PCR preparation buffer with nonionic detergents and Proteinase K (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 2.5 mM $MgCl_2$; 0.1 mg/mL gelatin; 0.45% NP40; 0.45% Tween 20; and 60 μg/mL freshly prepared proteinase K) [30]. PCR samples were incubated at 55°C for 1 hour and inactivated at 95°C for 10 minutes. The samples were then used as templates for PCR amplification. Primers were located in GC exon 9 and exon 11 (GC9: ACT TTG TCG ACA GTC CCA TC; GC11: CGC CAC AGG TAG GTG TGA AT). AmpliTaq and reagents were purchased from Perkin Elmer Cetus (Norwalk, CT). PCR conditions were set as follows: initial denaturation at 94°C for 5 minutes; total 30 cycles of amplification; each cycle with denaturation (93°C, 1 minute 30 seconds), annealing (65°C, 2 minutes), and elongation (71°C, 1 minute 30 seconds with 2-second extension in each additional cycle); final reaction at 65°C for 4 minutes, 71°C for 8 minutes, and slowly cooling to room temperature. Amplified products were loaded on 1% agarose gels. At the end of electrophoresis, the gel was illuminated on ultraviolet and then blotted onto Nytran membrane for Southern blot analysis using ^{32}P-labeled GC cDNA probes.

Northern blot analysis

RNA extraction and hybridization conditions were as described elsewhere [31].

GC enzymatic assay

CFU-GM colonies were pooled from methylcellulose culture. Cell lysates were prepared as previously described [32]. Briefly, cells were harvested, suspended in 100 μL potassium citrate/potassium phosphate buffer (pH 5.9) containing Triton X-100 (2 mg/mL), and disrupted by three cycles of freezing-thawing in an ethanol–dry ice bath and a 37°C bath. The cellular extracts were centrifugated in an Eppendorf microcentrifuge for 15 minutes at 4°C. The supernatant was used for the GC assay [19] and for the assessment of protein [33].

Giemsa and Wright staining of hematological slides

Cultured cells (1 to 4×10^4 in 0.5 mL) were mounted on a slide by using Cytospin 3 (Shandon, Pittsburgh, PA). The slide was then stained with the aerospray hematology slide stainer 7100 (Wescor, Logan, UT).

Results

Amplified high-titer viral producers improve transduction of hematopoietic cells

Three amphotropic retrovirus packaging cell lines, PA317 [34], PsiCRIP [26], and GP+envAm12 [35,36] were used to produce the GC-containing retroviral vector, LGSN, for infection of $3T3^{tk-}$ and BM $CD34^+$ cells. GP+envAm12 proved to be the most efficient to transduce hematopoietic cells and was used in all subsequent experiments. In an attempt to increase the viral titer of our vectors, we multiplied the proviral copy number within the GP+envAm12 packaging cells by repeated transductions of ecotropic vectors. A-LGSN vector was created by coculture of GP+envAm12/LGSN and GPE86/LGSN cells, and the A-LG4 producer cells were generated by repeated supernatant infection. Both producer clones contained more than five proviral copies as determined by Southern blot analysis (Figure 2). The copy number was determined by using an enzyme (*Nhe* I) that cuts the proviral DNA once and can distinguish between individual integration sites. The *Nhe* I that cuts both LTRs was also used to estimate the number of copies. The A-LGSN was titered on $3T3^{tk-}$ cells and gave a titer of 1×10^7, or approximately one order of magnitude higher than the single copy GP+envAm12/LGSN vector (1.5×10^6). The A-LG4 vector could not be titered by G418 resistance, but it did contain seven to eight proviral copies. When it was used to infect $3T3^{tk-}$ cells, the viral copy number in the target cells was more than one per cell, similar to the A-LGSN. We therefore assumed that the A-LG4 vector had a similar titer to the

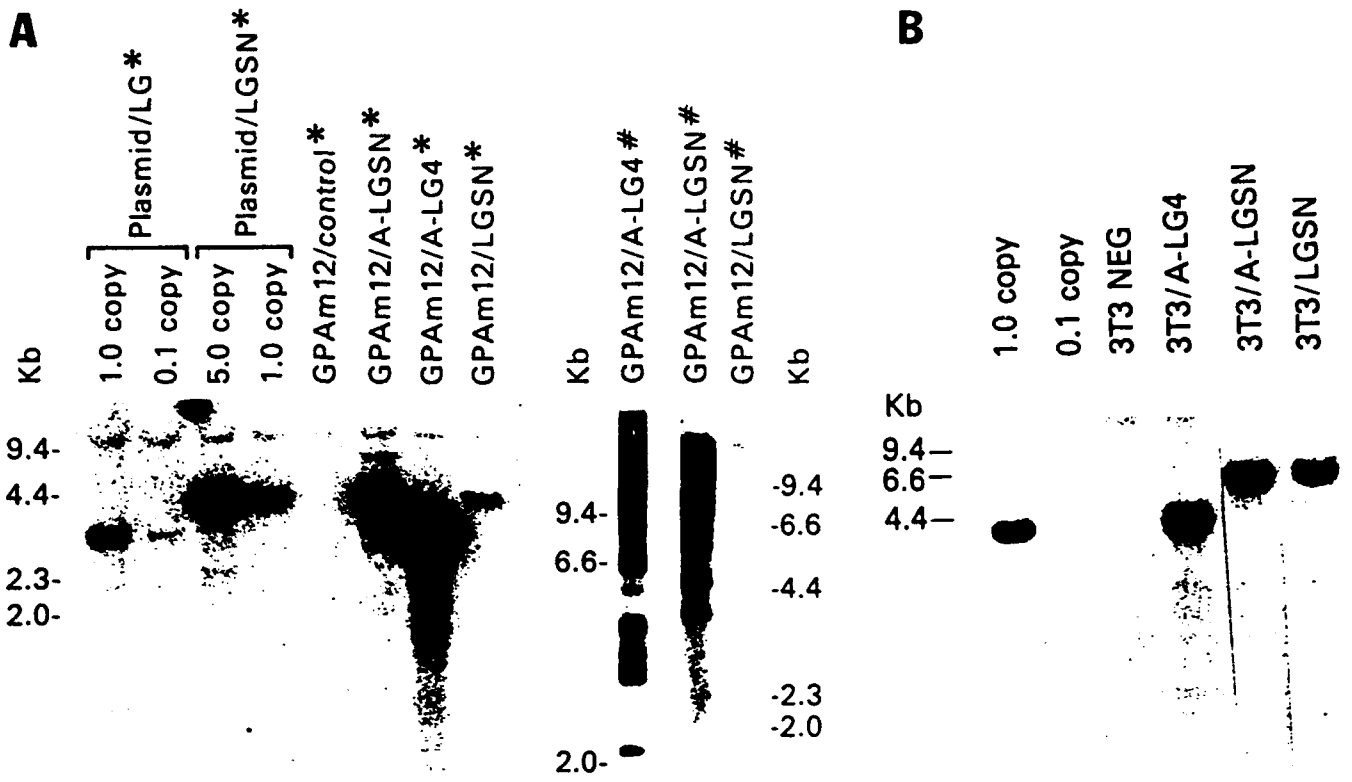

Fig. 2. A. Determination of proviral copy number (left) and integration number (right) of vector packaging cell lines. Genomic DNAs from GP+envAm12 (GPAm12) packaging cell lines were isolated and digested with *Nhe* I or *Xho* I. Digested DNA samples were fractionated in 1% agarose gel and transferred onto Nytran membrane, followed by Southern blot analysis. Plasmid/LG and plasmid/LGSN = mixed with genomic DNA and used as copy number control (copy/cell) as indicated; GPAm12/control = uninfected packaging cells; GPAm12/LGSN = unamplified LGSN; GPAm12/A-LG or A-LGSN = amplified (A) LG4 or LGSN.

* and # indicate DNAs digested with *Nhe* I and *Xho* I, respectively. Relatively short exposures were done to include signals from single and multiple copy producers on the same film. Bands representing mouse GC were therefore not seen as in longer exposures.

B. Determination of viral titers on $3T3^{tk-}$ cells: GPAm12 producers A-LG4, A-LGSN, and LGSN (single proviral copy) were titered on $3T3^{tk-}$ cells. DNA was extracted from $3T3^{tk-}$ cells 3 days after the infection and digested with *Nhe* I. A Southern blot was performed and the strength of the signal was compared with copy number controls (vector plasmid mixed with control $3T3^{tk-}$ cell DNA). The LGSN vector has a signal similar to that of the 1.0 copy control, but the amplified vectors A-LG4 and A-LGSN have a stronger signal and seem to have more than one copy per cell on average. The signal from the 0.1 copy is barely seen on this exposure. 3T3 neg are uninfected 3T3 cells.

Table 1. Transduction of human $CD34^+$ cells: comparison of producer cells with one or multiple proviral copies

Producers	Proviral copy number	3T3 titer	$CD34^+$ titer (PCR analysis)
GPAm12/LGSN	1	1.5×10^7	25% (9/36)
GPAm12/A-LGSN	>5	1.0×10^8	41% (23/56)[a]
GPAm12/A-LG	>5	[b]	42% (15/36)

[a]Data obtained from five independent experiments; all others, from three.

[b]3T3 titer similar to A-LGSN as determined by Southern blot.

A = amplified vector producer with multiple viral copies.

A-LGSN. Both of these vectors and all others used in this study were tested for helper virus by the marker rescue assay and were found to be free of helper virus.

The amplified vectors were compared to GP+envAm12/LGSN, a producer cell line that has a single proviral copy for transduction efficiency on $3T3^{tk-}$ cells (Fig. 2B) and human $CD34^+$ progenitor cells. The copy number was less in $3T3^{tk-}$ cells with the LGSN vector than with the amplified ones, A-LGSN and A-LG4 (Fig. 2B). The transduction efficiency of $CD34^+$ cells was increased to approximately 40% (an average of three to five experiments) using the amplified vector producer cells (Table 1).

Transduction of LTCIC from Gaucher patients

The high-titer vectors used here transduce hematopoietic progenitor cells from patients with Gaucher disease and normal individuals with similar efficiency, irrespective of whether the initial target cells are MNBM cells or $CD34^+$ BM cells (data not shown). Therefore we pooled data from MNBM cells and $CD34^+$ BM cells. Viral supernatants from the two amplified viruses, A-LGSN and A-LG4, were used to

Table 2. Transduction of bone marrow cells from Gaucher patients

	CFU-C efficiency					
	Patient I[a]		Patient II[a]		Patient III[b]	
	P/T[c]	%	P/T[c]	%	P/T[c]	%
Week 0						
Uninfected	0/5	0	0/5	0	0/5	0
A-LG4	ND	ND	6/10	60	3/10	30
A-LGSN	5/10	50	—	—	4/10	40
Week 3						
Uninfected	0/5	0	0/5	0	0/5	0
A-LG4	ND	ND	—	—	3/10	30
A-LGSN	2/10	20	6/10	60	5/10	50
Week 5						
Uninfected	0/5	0	0/5	0	0/5	0
A-LG4	ND	ND	1/10	10	4/10	40
A-LGSN	3/10	30	3/10	30	5/10	50

[a]Ficoll gradient–enriched BM cells were used.
[b]CD34⁺ BM cells were used.
[c]*Positive/total assessed by PCR analysis of individual colonies derived from clonogenic assays of LTBMC.*
ND = not done; — = technical failure.

infect either MNBM cells or BM CD34⁺ cells from patients with Gaucher disease and were cultured in vitro for 5 weeks. Cells were sampled at different time points. As shown in Table 2, analysis of clonogenic progenitors obtained immediately after infection or recovered from the 3-week-old LTBMC, revealed high gene transfer efficiencies, mostly ranging from 20 to 60% (an average of 40%). Significantly, 32% of CFU-GM progenitors recovered from the 5-week-old culture contained the recombinant GC sequence (295 bp) (Fig. 3), indicating that amplified viruses can efficiently transduce long-term bone marrow culture initiating cells using the supernatant infection protocol.

Correction of the enzyme deficiency in Gaucher LTCIC following gene transfer

To test for vector expression in the progeny of primitive hematopoietic cells, mRNA was isolated at week 5 of LTBMC from A-LGSN–transduced bone marrow cells of Gaucher patient I. As shown in Figure 4, the expected 5.3-kb transcripts from the transduced recombinant retroviral vector A-LGSN could be detected using the GC-specific probe labeled with ^{32}P-dCTP.

MNBM cells and BM CD34⁺ cells from Gaucher patients were cultured on irradiated heterologous stromal cells for 5 weeks after retroviral transduction. At week 0 (immediately after transduction), week 3, and week 5, cells were assayed for clonogenic progenitors, and after being in methylcellulose culture for 2 weeks, CFU-GM colonies were pooled to determine GC activity. The specific GC activity (nM/mg protein/min) from three different individuals is summarized in Table 3. All enzyme measurements were compared with the enzyme activity in normal control colonies (43 nM/mg protein/min, an average of several experiments). Considerable increases in GC activity of cells transduced by both A-LG4 and A-LGSN were seen in all three patient samples tested. These increases were seen at all time points sampled throughout the entire 5-week culture period. At 5 weeks postinfection, the enzyme levels in the progeny cells from progenitors harvested after 5 weeks of long-term culture were equal to or higher than levels found in asymptomatic heterozygous carriers (one exception: in patient II, vector LG-4 at 3 weeks was only 44% of control values). On average, the enzyme levels (A-LGSN) relative to control of normal BM cells were 84% at week 0, 70% at week 3, and 74% at week 5 (Table 3). Progenitors positive for the transferred GC gene were 45% (9/20) at week 0, 43% (13/30) at week 3, and 37% (11/30) at week 5 (Table 2), consistent with the level of enzyme activity, although the correlation was not perfect. The enzyme activity was measured in the progeny of clonogenic progenitors 7 weeks after the infection, not in the total nonadherent cell population as in a previous study [20]. The number of mature macrophages, harvested and dislodged from the LTC by pipette, increases with time (9% at week 0, 21% at week 3, and 29% at week 5 in one experiment), and macrophages tend to have higher GC enzyme activity per unit of protein than other hematopoietic cells (Correll et al., unpublished studies). Therefore the rise in enzyme activity will tend to be higher at 5 weeks than at week 0 when the gene transfer efficiency is the same at both time points and total nonadherent cells are measured. We did not compare the proportion of macrophages in the colonies derived from progenitors of LTC at weeks 0, 3, and 5 and can therefore not speculate whether

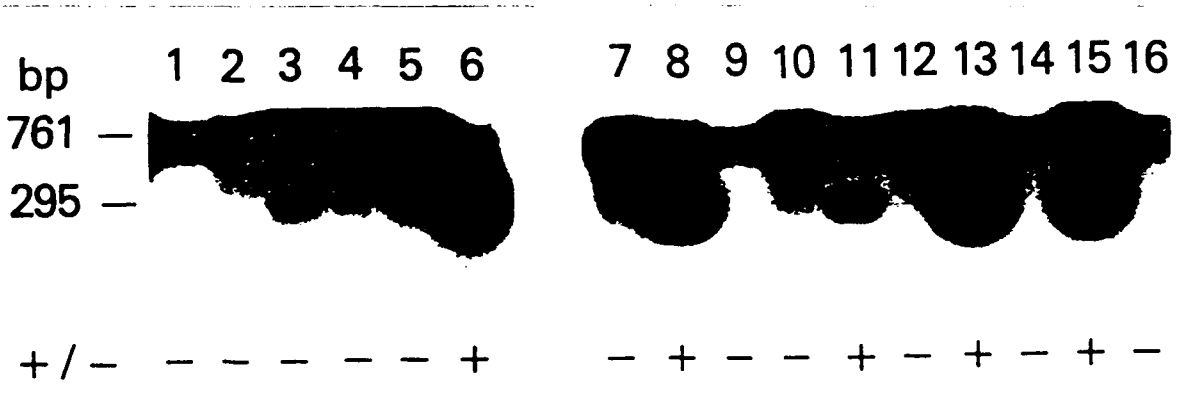

Fig. 3. Determination of GC gene transfer efficiency by PCR. CD34⁺ cells were cultured in the presence of irradiated heterologous feeders for 5 weeks in vitro and harvested for CFU-GM assay in methylcellulose medium. Well-isolated single colonies were picked and processed for PCR. Lanes 1–5: non-transduced cells; lane 6: positive A-LGSN control; lanes 7–16: A-LG4–transduced cells. 761 bp and 295 bp = amplified DNA fragments from the genomic DNA and the transduced vector, respectively. +/– = presence or absence of the 295-bp DNA fragment. Four of 10 colonies picked in this experiment were positive for the vector.

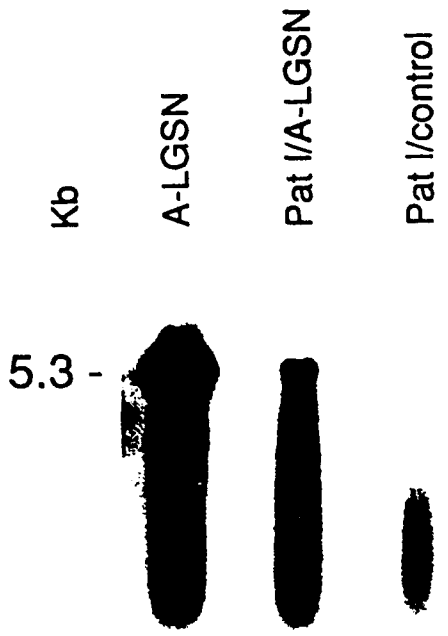

Fig. 4. Expression of GC cDNA in A-LGSN–transduced MNBM cells from Gaucher patient I at week 5. MNBM cells were isolated, infected, and cultured in the presence of irradiated heterologous feeder cells for 5 weeks in vitro, and RNA from the cells was examined by Northern blot analysis (probed using ^{32}P-labeled GC cDNA). A-LGSN = total cellular RNA extracted from a producer cell line A-LGSN; Pat I/A-LGSN and Pat I/control = RNA from A-LGSN–transduced and from the nontransduced BM cells of Patient I, respectively. The expected size of the transcript is 5.3 kb.

Table 3. GC activity of transduced BM cells from Gaucher patients

	nM/mg protein/min (avg ± SD)			
	Patient I	Patient II	Patient III[a]	Average
Week 0[b]				
Uninfected	11.7±0.8	17.8±0.7	17.1±1.1	15.6 (36%)[c]
A-LG4	ND	37.6±2.8	32.9±5.5	35.3 (82%)
A-LGSN	22.2±1.4	41.1±2.5	45.3±4.1	36.2 (84%)
Week 3				
Uninfected	13.1±2.7	9.1±1.2	12.4±0.4	11.5 (27%)
A-LG4	ND	22.6±1.4	25.4±1.0	24.0 (56%)
A-LGSN	26.8±2.3	30.8±3.9	32.5±3.4	30.1 (70%)
Week 5				
Uninfected	16.7±2.6	12.0±0.3	18.2±1.4	15.7 (36%)
A-LG4	ND	18.8±2.5	33.0±4.4	25.9 (60%)
A-LGSN	29.9±1.1	28.4±3.2	36.8±1.3	31.7 (74%)

[a]CD34$^+$ cells used in this experiment.
[b]GC activity measured in triplicate by pooling of CFU-GM colonies from one patient and SD calculated with n=3. GC activity in the normal control in the week 0: 43.03 nM/mg protein/min on average.
[c]Percentage in parentheses: GC activity relative to an average of the normal control.

differential composition of progeny cells in the colonies from different time points may have affected the enzyme values.

Discussion

We have efficiently transferred the human GC cDNA into mononuclear BM cells and CD34$^+$ BM cells from Gaucher type I patients using supernatants from the amplified amphotropic retroviral producer cell lines A-LGSN and A-LG4. These transduced cells initiated and maintained long-term bone marrow cultures in the presence of irradiated heterogeneous bone marrow fibroblast feeders, and sustained GC enzyme production was demonstrated until the culture was terminated at week 5. Since the enzyme activity was measured in pooled colonies derived from hematopoietic progenitors harvested after 5 weeks of long-term culture, we conclude that efficient transduction of LTCIC from Gaucher patients has been achieved and that the enzyme deficiency as measured in their progeny cells has been corrected.

Gene transfer into HSC has been problematic, since these cells are rare in BM [37] and mostly quiescent [38]. Several approaches have been developed to maximize transduction efficiency of the HSC, such as use of hematopoietic growth factors during infection [28,39–44], better vector design [9,45], the use of coculture instead of supernatant infection, amplification of copy number in producer cell lines [23,24,26], and purification of CD34$^+$ cells to increase the vector-to-target ratio [46]. To increase transduction efficiency of human hematopoietic cells without having to use a coculture procedure, we constructed amplified retroviral producer cell lines containing multiple proviral copies to increase viral titer. Our experiments clearly indicate that multiple viral copies in the producer cells will increase their ability to transduce human hematopoietic progenitors. The level of gene transfer efficiency into LTCIC achieved approximately 40%, similar to the percentage level of chimerism that exists in at least one Gaucher patient who has undergone transplantation and has improved clinically [47]. Our data suggest that efficient supernatant infection can be achieved with high-titer retroviral producer cells, consistent with a recent report by Hughes et al. [28]

Use of supernatant infection instead of coculture may be more easily justified in the setting of clinical gene therapy than cocultivation of human BM cells with murine retroviral producer cells. Techniques using transformed mouse cells in coculture with hematopoietic cells are not considered safe for human gene therapy, since a fraction of the mouse cells would likely be transplanted together with the hematopoietic cells of a patient and possibly pose complications to the patient. Although the use of autologous stromal cells to increase gene transfer efficiency by viral supernatants [21] is a possibility[1] it would be preferable and much simpler technically to use a stroma-free supernatant transduction protocol.

Nolta et al. [20] used a coculture transduction protocol to correct the enzyme deficiency in hematopoietic cells of a Gaucher patient. Nonadherent cells from a long-term culture were harvested 4 weeks after the infection to demonstrate the

[1]Recombinant DNA Advisory Committee (RAC) has already approved supernatant transduction protocol with autologous stroma. The RAC has also approved the use of packaging cells with multiple proviral copies to increase viral titer in supernatants for clinical gene therapy experiments including a supernatant transduction protocol using an amplified LG vector producer cell line from this laboratory.

correction of the enzyme deficiency compared to control cultures. The enzyme was measured in all the nonadherent cells in the culture, including all the mature cells derived from progenitors that are less primitive than LTCIC. It was therefore impossible to demonstrate decisively successful gene transfer into LTCIC. Our goal was to demonstrate whether correction of the enzyme deficiency in hematopoietic cells of Gaucher patients could be achieved using a clinically acceptable supernatant transduction protocol and whether this protocol could be used to transduce LTCIC efficiently and correct the enzyme deficiency in their progeny cells. Therefore we measured enzyme activity in hematopoietic colonies derived from progenitors that were harvested from the long-term culture up to 5 weeks following the transduction. Enzyme measurements were therefore done after culture up to 7 weeks (2 weeks in methylcellulose) after the transduction. The progeny cells of the last time point should therefore all be derived from LTCIC. Correction of the enzyme deficiency was demonstrated at all time points (Table 3), and the enzyme measurements correlated relatively well with the transduction efficiency as determined by PCR analysis of CFU-GM colonies.

It is encouraging that the overall enzyme activities following gene transfer are well above the average enzyme activity of asymptomatic heterozygotes and this includes the last time point designed to measure gene transfer into LTCIC. However, it is unclear whether this would be sufficient to cure Gaucher disease. Asymptomatic heterozygotes have approximately 50% of the normal GC enzyme activity in every cell. In our experiment, 40% of the cells contain GC activity that is at normal or even higher levels, while the remaining 60% are genetically unmodified and have low enzyme activity. Since the enzyme is a membrane-bound glycoprotein that is not secreted from the cell to any extent, it is unlikely that the enzyme produced in corrected cells will have direct effects on uncorrected cells. Despite these considerations, it is interesting to note that the Gaucher patients who have undergone allogeneic transplantation with incomplete engraftment resulting in stable chimerism have improved clinically [47], but detailed studies on the extent of their chimerism, resolution of pathological changes, and clinical follow-up have not been reported to our knowledge. It is possible that lipid-laden macrophages may eventually die and the lipid may then be engulfed by normal or gene-corrected macrophages. If that is the case, the percentage of gene-corrected macrophages required for clinical improvement is considerably less than 100%.

While the LTCICs that have been transduced are primitive multipotential cells, they cannot be claimed to be repopulating hematopoietic stem cells. To assay the latter cells, transplantation studies have to be performed in xenograft models or large animals or in phase I clinical trials. These studies may help to answer questions not easily addressed by in vitro experiments.

In summary, we have created high-titer amphotropic retroviral producer cell lines using either coculture or repetitive supernatant infection. Our results demonstrate that supernatants from these amplified producer cell lines can efficiently transduce LTCIC from bone marrow of Gaucher patients and that potentially therapeutic levels of GC enzyme production are achieved in their progeny cells.

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