Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2025–2030, March 1996
Medical Sciences

Long-term *in vitro* correction of α-L-iduronidase deficiency (Hurler syndrome) in human bone marrow

(gene therapy/mucopolysaccharidosis type I/autosomal recessive)

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Communicated by J. B. Gurdon, Wellcome/Cancer Research Campaign Institute, Cambridge, United Kingdom, October 16, 1995 (received for review July 10, 1995)

ABSTRACT **Allogeneic bone marrow transplantation is the most effective treatment for Hurler syndrome but, since this therapy is not available to all patients, we have considered an alternative approach based on transfer and expression of the normal gene in autologous bone marrow. A retroviral vector carrying the full-length cDNA for α-L-iduronidase has been constructed and used to transduce bone marrow from patients with this disorder. Various gene-transfer protocols have been assessed including the effect of intensive schedules of exposure of bone marrow to viral supernatant and the influence of growth factors. With these protocols, we have demonstrated successful gene transfer into primitive CD34$^+$ cells and subsequent enzyme expression in their maturing progeny. Also, by using long-term bone marrow cultures, we have demonstrated high levels of enzyme expression sustained for several months. The efficiency of gene transfer has been assessed by PCR analysis of hemopoietic colonies as 25–56%. No advantage has been demonstrated for the addition of growth factors or intensive viral exposure schedules. The enzyme is secreted into the medium and functional localization has been demonstrated by reversal of the phenotypic effects of lysosomal storage in macrophages. This work suggests that retroviral gene transfer into human bone marrow may offer the prospect for gene therapy of Hurler syndrome in young patients without a matched sibling donor.**

Mucopolysaccharidosis (MPS) type I is a lysosomal storage disorder caused by a deficiency of the enzyme α-L-iduronidase (EC 3.2.1.76; IDUA) and is characterized by accumulation of the glycosaminoglycans dermatan and heparan sulfate (1, 2). The clinical phenotype can be very variable (3). In the most severe form of Hurler syndrome, individuals show progressive neurological dysfunction, have multiple skeletal and soft tissue abnormalities, and generally die within the first decade. In the relatively mild Scheie syndrome, patients present much later in life, may have detectable but reduced (1–5% normal) levels of IDUA activity, much milder symptoms with no neurological dysfunction, and live into adulthood (4).

As yet there is no specific therapy for the disorder apart from allogeneic bone marrow transplantation. This approach relies on the donor bone marrow producing and secreting functional enzyme, which is subsequently taken up by and targeted to the lysosomes of other cell types. This approach has been shown to have modest success, particularly when the transplant is undertaken before the age of 18 months. After successful transplantation, significant and progressive orthopedic problems remain and are the main problem in clinical management. The ultimate neurological prognosis remains guarded, but a clear slowing of neurological deterioration has been documented in patients transplanted at an early age (5–8). Unfortunately, allogeneic transplantation is limited both by the availability of donors and by significant morbidity in matched unrelated donor transplants.

The cDNA and gene for IDUA have been cloned (9, 10) and expression of the cDNA *in vitro* in fibroblasts derived from a patient with Hurler syndrome leads to correction of the enzyme deficiency, localization of functional enzyme to the lysosomes, and reduction in the accumulation of sulfated glycosaminoglycans by corrected cells (11). Infusion of purified human IDUA in a canine model of Hurler syndrome led to normalization of lysosomal storage in some soft tissues, but not in brain, heart valves, or cornea (12).

In the present report, we provide evidence that the IDUA cDNA can be transferred into primitive hemopoietic cells, that enzyme levels can be corrected, and that phenotypic correction of the deficiency occurs *in vitro*.

MATERIALS AND METHODS

Preparation of Virus Producers. pLid (Fig. 1) was created by inserting the human IDUA cDNA into plasmid pLX, derived from the vector pLNCX (13) by deleting the cytomegalovirus and neomycin-resistance sequences. This was used to derive amphotropic retroviral producers, using GP+envAm12 cells (14) and standard methodology (15). The clone of amphotropic packaging cells that led to the highest levels of IDUA in target Hurler B cells after cocultivation was chosen for further study and named GPaLid. To estimate the titer of retrovirus produced by this clone, conditioned medium was used to transduce Hurler B cells in 24-well plates in a limiting dilution assay. This was compared with virus supernatants from a second IDUA producer that produced the LidSN virus expressing both IDUA and neomycin phosphotransferase (Fig. 1). By obtaining a comparative titer from the Lid and LidSN virus producers and obtaining a G418 titer for LidSN on 3T3 cells, it was calculated that GPaLid produced the equivalent of 1–3 × 10^6 Geneticin transfer units per ml.

Transduction and Maintenance of Long-Term Bone Marrow Cultures (LTBMCs). After local ethics committee approval and informed consent, bone marrow was obtained from patients with Hurler syndrome and from healthy individuals. Samples were depleted of erythrocytes by gravity sedimentation through methylcellulose (0.1%, 30 min) and LTBMCs

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Abbreviations: LTBMC, long-term bone marrow culture; LTBMCM, LTBMC medium; MPS, mucopolysaccharidosis; IDUA, α-L-iduronidase; IL, interleukin; LTCIC, long term culture initiating cell.
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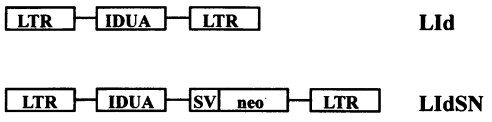

FIG. 1. Retroviral vectors expressing human IDUA. Retroviral expression vectors pLid and pLidSN express IDUA and both IDUA and neomycin phosphotransferase, respectively.

were initiated by seeding 2×10^7 cells per 25-cm^3 flask in LTBMC medium [LTBMCM; Iscove's medium (GIBCO) containing 10% (vol/vol) fetal calf serum, 10% (vol/vol) horse serum (Flow Laboratories), and 5×10^{-7} M hydrocortisone (Sigma)] (16). The cells were plated into 25-cm^2 flasks in 5 ml of LTBMCM (for nontransduced controls) or in LTBMCM conditioned by cocultivation with GPaLid cells.

A number of factors were studied to determine the optimal conditions for gene transfer. To examine the effects of growth factors, LTBMCM or virus supernatant was supplemented with endotoxin-free human recombinant interleukin (IL) 3 (2 ng/ml), human recombinant IL-6 (100 units/ml), and human recombinant stem cell factor (10 ng/ml). To investigate the effects of intensive exposure regimes, cultures were exposed to medium/virus supernatant once daily or five times daily for 4 days after inception. Twenty-four hours after the final addition of medium/virus, nonadherent cells were removed from the flasks, centrifuged ($800 \times g$ for 6 min; cells from separate flasks were centrifuged separately), resuspended in 10 ml of fresh medium, and replaced in their original flasks.

Subsequently, all cultures were maintained by feeding once weekly, removing 5 ml of medium and cells, and replacing this with 5 ml of fresh LTBMCM. At later time points, to obtain sufficient cells for analysis, cultures were fed by the removal and addition of 10 ml of medium.

Isolation and Culture of Enriched Progenitor (CD34$^+$) Cells from Long-Term Cultures. Skin fibroblasts from a patient with severe Hurler syndrome were grown to confluence in 24-well plates in DMEM/10% (vol/vol) newborn calf serum. On day 7 after inception, cells from long-term cultures of normal bone marrow, mock-transduced Hurler bone marrow, and virus-transduced (once daily for 5 days, no growth factors) Hurler bone marrow were harvested, and CD34$^+$ cells were isolated by using the MiniMACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). After isolation, 2500 CD34$^+$ cells were seeded per well of feeder fibroblasts, in LTBMCM/IL-3/IL-6/stem cell factor as described above. At various times after seeding, hemopoietic cells were harvested from wells for IDUA assay.

Efficiency of Gene Transfer. To determine the frequency of gene transfer to hematopoietic progenitors, the IDUA cDNA was assayed by using PCR in colonies derived from the nonadherent layer. Hemopoetic progenitor colonies were obtained by plating cells at 10^5 cells per ml in methylcellulose as described (16). After 14–18 days, colonies were picked into 50 μl of 50 mM KCl/10 mM Tris, pH 8.3/bovine serum albumin (0.1 mg/ml)/0.45% Tween 20/0.45% Nonidet P-40/2.5 mM $MgCl_2$/each PCR primer at 2 μM and flash-frozen in liquid nitrogen. The primers chosen for PCR analysis (see Fig. 4) define an expected product length of 113 bp from the cDNA but are separated by a 13-kb intron in the genomic sequence (10). For analysis, 50 μl of the above mixture containing 20% (vol/vol) dimethylsulfoxide, all four dNTPs (each at 200 μM), and 0.25 unit of *Taq* polymerase was added. Cycle conditions were 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Typically 40 cycles of PCR were performed, the products were analyzed on a 2% agarose gel in 0.5 × TBE and subjected to Southern blot analysis (17) with IDUA cDNA by using the high SDS method (18).

Analysis of IDUA Expression Level. IDUA activity of cell homogenates and culture medium was assayed essentially as described (19), by using 4-methylumbelliferyl α-L-iduronide (Calbiochem) as substrate. Cell pellets were suspended in 50–200 μl of 0.1% Triton X-100 and disrupted by five cycles of freezing and thawing. Supernatants obtained after centrifugation were used for enzyme assay. Culture medium was assayed directly.

A 20-μl sample was incubated with 20 μl of 1.5 mmol of substrate in 0.4 M sodium formate (pH 3.5) containing 155 mM NaCl at 37°C for 0.5–4 h. Reactions were terminated by addition of 2 ml of 0.2 M sodium carbonate (pH 9.5) and the fluorescence produced was measured by using a LS2-B fluorimeter (Perkin–Elmer).

Sulfate Sequestration Assay. LTBMCs were exposed to $^{35}SO_4$ in sulfate-free LTBMCM at 10 μCi/ml for 48 h. Cultures were then refed with conventional LTBMCM and incubated for 4 weeks with weekly feeding to chase radioactivity from non-glucosaminoglycan sources. After this time, adherent and nonadherent cell fractions were harvested, trypsinized to remove external glucosaminoglycans, washed in PBS, and then lysed by freezing and thawing in PBS. Subsequently, the ^{35}S content of samples was determined by scintillation counting.

RESULTS

Expression of IDUA in Control and Virus-Treated Cultures. Fig. 2*a* shows the levels of IDUA measured in nonadherent cells from LTBMCs. In LTBMCs from normal bone marrow, the level of IDUA found was initially low (1–3 nmol per h per 10^6 cells) and rose to a plateau of 10–30 nmol per h per 10^6 cells. On the other hand, over the 20-week course of the

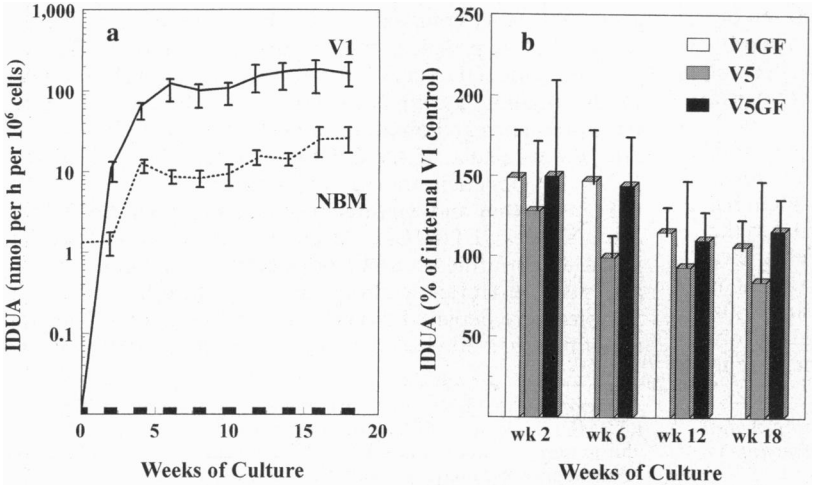

FIG. 2. Levels of IDUA in LTBMC. (*a*) IDUA levels in control untransduced Hurler LTBMC (■), normal LTBMC (NBM, dashed line), and Hurler LTBMC exposed to virus once daily in the absence of growth factors (V1, solid line). Data are expressed as the mean ± SEM; data are from seven experiments. (*b*) IDUA levels in cultures exposed to virus five times a day in the absence of growth factors (V5), once a day in the presence of growth factors (V1GF), or five times a day in the presence of growth factors (V5GF). Data are expressed as the percentage of internal V1 control (mean ± SEM); data are from six (V1GF), three (V5), and two (V5GF) experiments.

experiments, no detectable IDUA activity was found when cells from LTBMCs derived from seven Hurler syndrome patients were analyzed.

Exposure of LTBMCs from Hurler syndrome patients to retrovirus carrying the IDUA cDNA, once a day in the absence of added growth factors (group V1), resulted in expression of IDUA activity in these cultures, which was maintained for the life of the cultures. As in normal cultures, the virus-treated Hurler syndrome cultures initially showed low levels of IDUA, which increased over the first few weeks of culture to a plateau. However, the mean levels of IDUA detected in virus-treated cultures exceeded those found in normal LTBMCs by 10-fold. To test whether the use of growth factors or of more intensive protocols of virus exposure would lead to higher or more sustained expression of IDUA in patient bone marrow, cultures were exposed to virus either once a day in the presence of IL-3, IL-6, and stem cell factor (group V1GF) or five times a day in the presence (group V5GF) or absence (group V5) of these growth factors. Fig. 2*b* shows the levels of IDUA in these cultures expressed as a percentage of the levels found in V1 cultures. At early time points (weeks 2 and 4), the presence of growth factors during virus exposure seemed to lead to a modest increase in IDUA in the LTBMCs. At later time points, however, there was little if any difference in the levels of IDUA seen in cultures exposed to virus under the various conditions.

To test whether IDUA was being secreted by the cells of the LTBMCs, cell-free medium from LTBMCs was assayed for IDUA. The results are summarized in Fig. 3. Medium from control untransduced Hurler cultures contained no detectable IDUA activity. LTBMCs derived from unaffected individuals showed the presence of IDUA in the extracellular medium. Similarly, LTBMCs that had been exposed to Lid virus and contained detectable levels of IDUA in the nonadherent cell fraction also showed significant amounts of extracellular IDUA activity. As with the levels found in cells from these cultures, secreted IDUA was around 10 times that from normal LTBMCs. The use of growth factors did not confer any increased secretion of IDUA in virus-treated cultures. Neither did the use of intensive protocols of virus exposure (data not shown).

PCR Analysis of Colonies from LTBMCs. Having shown successful transfer and expression of the IDUA gene, we next determined the transfection frequency into primitive multipotent hemopoietic cells. To do this, mixed colony-forming cell assays were performed and colonies were picked for determination of the presence of the IDUA cDNA by PCR analysis. Fig. 4 shows the results of PCR analysis of colonies derived from Hurler LTBMCs 1 week after their final exposure to virus. A significant number of the colonies scored positive for the presence of the IDUA cDNA, whereas colonies derived from control cultures were negative.

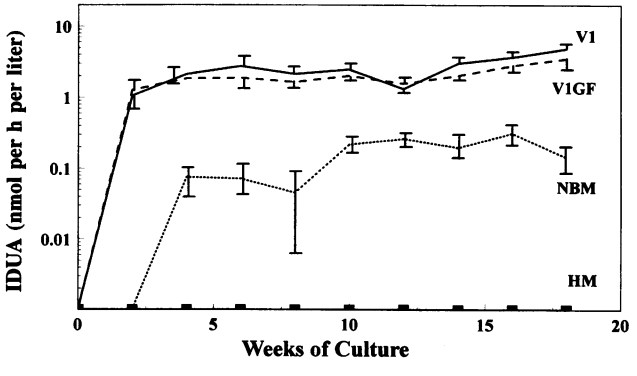

FIG. 3. Levels of IDUA in medium from LTBMC. Levels of IDUA detected in the extracellular medium of LTBMCs. Control, untransduced Hurler LTBMC (HM, ■), normal LTBMC (NBM, dotted line), Hurler LTBMC exposed to virus once daily in the absence of growth factors (V1, solid line), and Hurler LTBMC exposed once daily in the presence of growth factors (V1GF, dashed line) are shown.

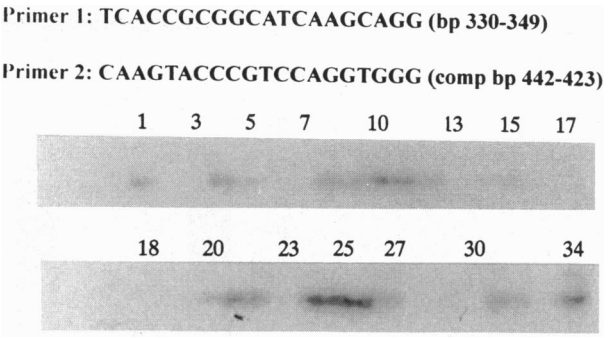

FIG. 4. PCR analysis of hemopoietic colonies. Forward (primer 1) and reverse (primer 2) PCR primers for detection of IDUA cDNA. PCR signals obtained from 10^3 GPaLid packaging cells (positive control, lane 34), hemopoietic colony from nontransduced cells (negative control, lane 17), and hemopoietic colonies derived from virus-transduced Hurler LTBMC after retroviral transduction (lanes 1–16 and 18–33).

The results of PCR analyses performed on colonies from a number of experiments are summarized in Table 1. One week after transduction, 20–50% of the colonies analyzed contained the IDUA cDNA. Analysis of colonies derived from cultures 5 and 9 weeks after exposure to retrovirus indicated that a substantial (up to 60%) proportion of these cells also contained the IDUA retroviral vector. As with analysis of the IDUA expression in the cultures, there appeared to be no obvious advantage in the use of growth factors or intensive transduction schedules, as judged by transduction frequency.

Expression of IDUA in CD34$^+$ Cells from Long-Term Cultures. Primitive hemopoietic (CD34$^+$) cells were isolated from virus-treated and control Hurler syndrome long-term cultures and from normal long-term cultures and cultured in 24-well plates for several weeks in the presence of growth factors and of IDUA-deficient skin fibroblasts derived from a patient with Hurler syndrome. This was done to support the continued growth and amplification of the hemopoietic cells. Fig. 5 shows the percentage of wells that contained detectable IDUA activity during the monitoring period. Between 40% and 60% of the wells were found to have detectable enzyme, with 11–20% of the wells showing enzyme levels at or approaching the normal range (>1 nmol per h per 10^6 cells). When the average levels of expression of IDUA in the wells were assessed with time (Fig. 6), the levels of enzyme expression in virus-transduced CD34$^+$ cells from Hurler syndrome cultures were stably maintained. These levels constituted 9.2–35.5% of those found in normal CD34$^+$ cells. In contrast, no detectable activity was found in CD34$^+$ cells from control untransduced Hurler syndrome cultures.

Table 1. PCR analysis of hemopoietic colonies

Experiment	Group	No. PCR-positive colonies/ total no. colonies		
		Week 1	Week 5	Week 9
HMVII	V1	1/17 (6)		
	V1GF	8/20 (18)		
	V5	11/23 (43)		
	V5GF	2/16 (12)		
HMVI	V1	8/20 (40)	9/16 (56)	
	V1GF	1/4 (25)	9/16 (56)	
	V5	9/22 (41)	4/20 (20)	
	V5GF	13/25 (52)	6/12 (50)	
HMIX	V1	5/23 (22)		12/20 (60)
	V1GF	13/23 (56)		11/20 (55)

Data in parentheses are percent of total colonies.

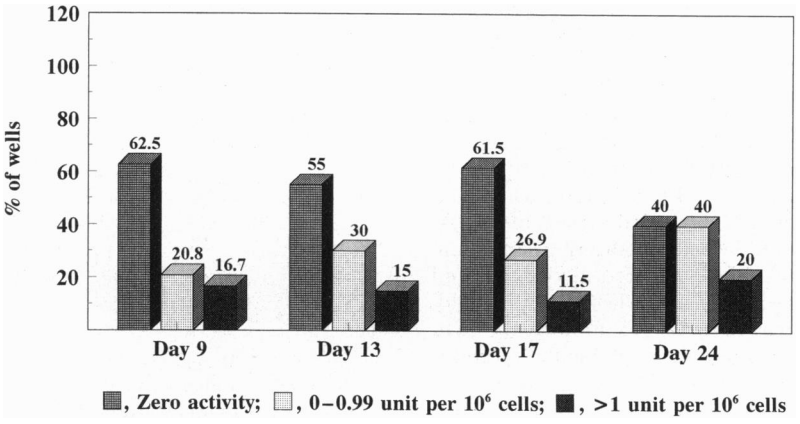

FIG. 5. Expression of IDUA in CD34+ progenitor cells. CD34+ cells seeded onto Hurler fibroblasts and grown for indicated times before harvesting for IDUA assays. Proportions of wells at indicated times exhibiting undetectable, low (0–0.99 nmol per h per 10^6 cells), or high (>1 nmol per h per 10^6 cells) levels of IDUA.

Expression of IDUA in LTBMC Cells Reverses the Phenotypic Effects of Hurler Syndrome. When the morphology of cells from the LTBMCs was analyzed, macrophages from untransduced Hurler syndrome cultures showed lysosomal distension and the characteristic deposits of glucosaminoglycans (Fig. 7 *c* and *d*). Macrophages taken from Hurler syndrome cultures that showed IDUA expression as a result of retroviral gene transfer did not exhibit lysosomal distension (Fig. 7 *e* and *f*). Instead their morphology more closely resembled that of macrophages from unaffected individuals (Fig. 7 *a* and *b*), with complete clearance of deposits.

To assess the uptake and sequestration of sulfate by corrected and uncorrected Hurler syndrome bone marrow, cultures were exposed to ^{35}S in sulfate-free medium for 48 h and then cultured for 4 weeks in sulfate-containing medium to allow turnover of sulfate pools in the cells. After this time, cells were lysed and their total ^{35}S load was measured by scintillation counting. As shown in Fig. 8, cells from the adherent and nonadherent fractions of a control nonvirus-treated Hurler syndrome LTBMC showed significant levels of ^{35}S sequestration. In contrast, however, the levels of ^{35}S detected in virus-treated Hurler LTBMC matched the low levels seen in a normal LTBMC.

DISCUSSION

Hurler syndrome, in common with a number of similar single gene disorders, collectively termed the MPSs, is a lysosomal storage disease that results in accumulation of waste products within the lysosomes of all tissues in affected individuals. A wide range of clinical symptoms are manifested, including in some instances, progressive neurological decay as a result of lysosomal storage within the central nervous system. Where neurological effects are manifested, infusion of recombinant enzyme will be of little benefit due to lack of transfer of infused enzyme across the blood–brain barrier. Indeed, other than palliation, there is currently no therapeutic option for the MPS other than allogeneic bone marrow, which can offer transfer of enzyme across the blood–brain barrier in the form of donor macrophages that can replace the host microglial cells (20). Unfortunately, this approach is limited both by the availability of donors and by significant morbidity in matched unrelated donor transplants.

Because of this, a number of groups have been evaluating the possibilities for genetic therapy of the MPS. The genes defective in a number of syndromes have been isolated and tested for their ability to reverse the disease phenotype in fibroblast or immortalized cell lines from patients with various types of MPS (11, 21–23). Such experiments document effective enzyme production and aleviation of phenotypic symptoms of disease. Indeed for the treatment of some disorders that do not cause neurologic degeneration, *in vivo* expression and secretion of protein products by genetically manipulated fibroblasts may be able to provide alleviation of the symptoms of disease (24). However, where there is neurological involvement (as is the case in Hurler syndrome), the "fibroblast factory" approach suffers from the same problem as those based on infusing enzymes or protein products—namely, the problem of

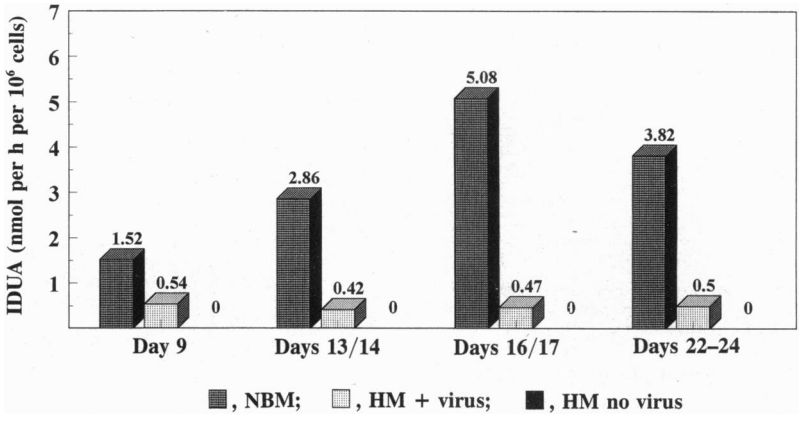

FIG. 6. Expression of IDUA in CD34+ progenitor cells. CD34+ cells seeded onto Hurler fibroblasts and grown for indicated times before harvesting for IDUA assays. Mean levels of IDUA at indicated times in wells containing normal bone marrow (NBM), untransduced Hurler bone marrow (HM), or retrovirally transduced Hurler bone marrow (HM + virus).

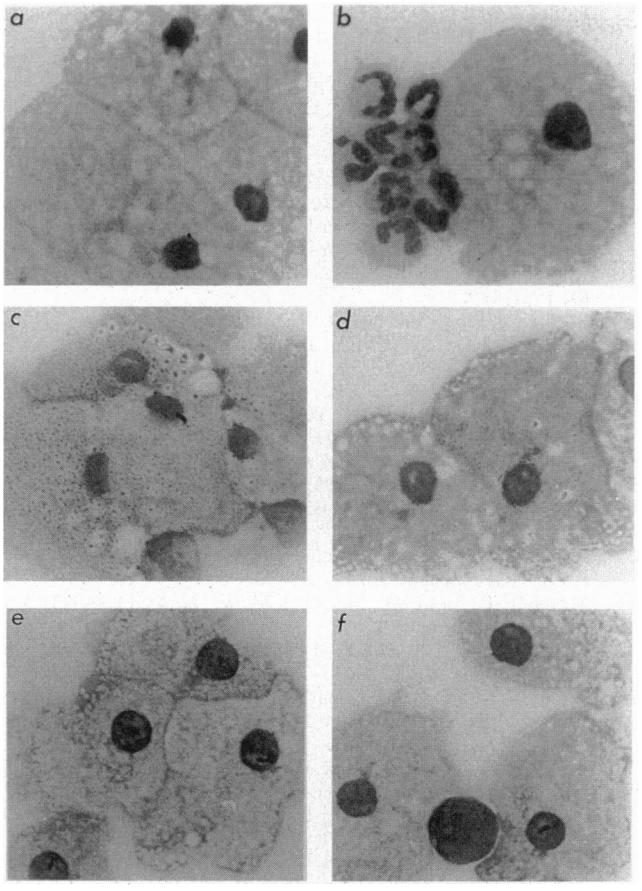

FIG. 7. Morphological correction of macrophages from Hurler syndrome bone marrow cultures. May–Grunwald/Giemsa-stained cytospin preparations from 6-week-old long-term cultures of normal bone marrow (*a* and *b*), untransduced Hurler bone marrow (*c* and *d*), and retrovirally transduced Hurler bone marrow (*e* and *f*).

the blood–brain barrier. Thus, the use of genetically modified fibroblasts is unlikely to present a realistic therapeutic option where neurological disease is involved.

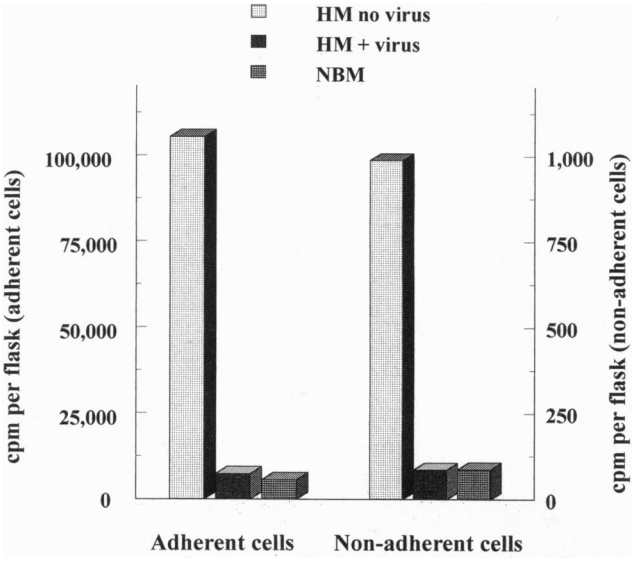

FIG. 8. Sequestration of ^{35}S by LTBMC. Radioactivity retained by control, untransduced Hurler (HM no virus), virus-transduced Hurler (HM + virus), and normal (NBM) LTBMCs. Data are expressed as total cpm per flask.

For these reasons, we have been evaluating the possibility of using genetically modified bone marrow to deliver IDUA to the cells of individuals with Hurler syndrome, an approach that has shown some efficacy in murine models of a related disorder (25, 26). This approach has several attractions over current allogeneic transplant approaches and over the use of genetically modified fibroblasts. (*i*) The approach is available to individuals without donors and the risks of graft vs. host disease are abolished. (*ii*) As with donor macrophages after allogeneic bone marrow transplantation, genetically modified macrophages derived from transduced hemopoietic progenitor and stem cells can replace the incumbent microglial population and so deliver enzyme to the central nervous system.

As a prerequisite to embarking on clinical trials of genetically modified bone marrow transplantation, it is first necessary to demonstrate that the gene is targeted to the most primitive bone marrow progenitors (stem cells), as it is these cells that sustain hemopoiesis over the lifetime of an individual. To achieve high levels of enzyme expression, it is also advantageous to target stem cells with high efficiency. These issues can present problems as retroviruses only successfully infect cycling cells (27) and the majority of bone marrow stem cells are out of cycle (28).

The LTBMC system allows us to address some of these issues. When bone marrow is initially sampled and the LTBMC is established, it contains a mixture of pluripotent stem cells, lineage committed cells, and stromal elements. Over a period of weeks, a stromal layer is laid down and the self-renewing marrow-repopulating stem cells associate with the stromal layer. These cells sustain hemopoiesis giving rise to committed progeny in free-cell suspension. Importantly, committed progenitor cells sampled in the initial bone marrow aspirate undergo clonal extinction over a period of 6–8 weeks, so that after this time point the population of cells isolated from the nonadherent layer are derived from the primitive long term culture initiating cell (LTCIC) population (29, 30), which although clearly an *in vitro*-defined population af cells, exhibit many of the characteristics of stem cells. Continued expression of the transferred gene in hemopoietic cells after 6 weeks indicates successful transduction of the LTCIC. Of equal importance in the context of reinfusion and engraftment of the genetically manipulated cells described in our current study is that we have successfully used bone marrow cells (grown for up to 2 weeks in LTBMC) to regenerate hemopoiesis in patients with a variety of hemopoietic malignancies (31, 32).

The present data clearly show that expression can be achieved well beyond the critical 6-week period at a level equal to or in excess of levels found in normal bone marrow donors. Although this supports the view that the stem cells have been successfully targeted, because the enzyme is secreted, the high levels of enzyme activity in the nonadherent cellular population could result from lysosomal targeting of secreted enzyme from retrovirally transduced stromal cells or from a combination of stromal secretion and gene expression in LTCIC and their progeny. However, the PCR analysis indicates that a substantial proportion of LTCIC progeny (as measured by colony forming cells at 5–9 weeks) contain the IDUA gene. This would support the successful transfer of the gene into target cells. More importantly, isolation and expansion of primitive $CD34^+$ cells from cultures after the transduction procedure indicated that these also expressed IDUA, at levels between 10% and 35% of those found in $CD34^+$ cells from bone marrow of unaffected individuals. Moreover, these levels of enzyme were stably maintained. The clinical relevance of these levels of enzyme expression is of obvious importance. In this context, it is worth noting that individuals with the mild form of MPS-I (Scheie syndrome) express levels of functional IDUA of the order of 1–5% of that found in normal individuals. In Scheie syndrome, there is no neuronal dysfunction and reduced skeletal and visceral defects, and affected individuals

survive into adulthood. Given this, it would seem that the levels of IDUA reported in $CD34^+$-derived hemopoietic cells in this study might be sufficient to give alleviation of many of the aspects of severe Hurler syndrome.

The efficiency of gene transfer is at least as good as that reported by many other groups (33–35), and in our system, growth factors and marrow manipulation do not appear to increase the efficiency of gene transfer. Thus a relatively simple transduction schedule, avoiding manipulation/conditions that might compromise the engraftment potential of the marrow, facilitated good gene transfer and enzyme expression.

In summary, we have shown that it is possible to obtain efficient transfer and functional expression of the IDUA gene in Hurler bone marrow and to sustain enzyme expression over many months. Clinical trials of this approach should soon be underway and will allow closer examination of the potential of genetically manipulated bone marrow for the treatment of metabolic disorders involving neurological dysfunction.

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