Retroviral-Mediated Gene Transfer in Human Bone Marrow Cells Grown in Continuous Perfusion Culture Vessels

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Hematopoietic stem cell gene therapy holds the promise of being able to treat a variety of inherited and acquired diseases of the hematopoietic stem cell. However, to date, genetic modification of the human hematopoietic stem cell has been relatively inefficient. Here, we report the results of using a bioreactor system to expand hematopoietic cells after a brief retrovirus infection using a high titer, replication defective virus encoding for murine CD18. The retrovirus transduced culture continued to produce genetically modified hematopoietic progenitors for up to 6 weeks, the duration of the culture period. Up to one-third of the long-term culture initiating cell (LTC-IC) are genetically modified by the culture conditions. Murine CD18 can be expressed on the cell surface of up to 20% of the mature cells generated by the culture system, suggesting that clinically significant levels of gene transfer may be occurring. These results demonstrate the feasibility of using continuous perfusion bioreactors as a method of efficiently modifying human hematopoietic stem cells.

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Molecular Biology has opened the way for the introduction of new genetic material into human cells.1-3 Gene therapy using hematopoietic stem cells as the therapeutic target cell population holds great promise to dramatically improve treatment of both hereditary and acquired diseases that involve blood cells. For example, hereditary hemoglobinopathies such as the thalassemias or sickle cell anemia could be corrected by the introduction of a gene that expresses large amounts of fetal hemoglobin. Acquired disorders, such as acquired immunodeficiency syndrome (AIDS), could potentially be treated by genetically engineered resistance to human immunodeficiency virus (HIV) into the hematopoietic stem cell.

Previously, we have shown that successful human long-term bone marrow culture (LTBMC) depends on a combination of culture conditions, including rapid media exchange, media supplemented with hematopoietic growth factors (HGFs), and development of a suitable microenvironment.4,5 Optimal growth conditions are achieved by the development of an adherent stromal cell layer, which provides anchorage sites and membrane bound and soluble growth factors.6 Supplementing media with a combination of several HGFs produces synergistic activities that affect stromal, progenitor, and stem cells.7,8 Rapid media exchange provides a relatively constant level of HGFs, as well as maintaining constant serum turnover, both of which more closely mimic the in vivo milieu. These factors are combined effectively in a recently reported culture system using continuous perfusion of closed cell culture chambers.12 LTBMC may now expand long-term culture initiating cells (LTC-IC) threefold to sevenfold in 14 days and colony-forming unit granulocyte macrophages (CFU-GMs) 8- to 21-fold in the same period. This represents a significant improvement over earlier culture techniques.

The best studied and most efficient method to transfer new genetic material into hematopoietic cells uses replication-deficient murine retroviruses. Use of these vectors in conjunction with a culture system that drives the cycling of hematopoietic stem cells should increase the long-term success of retroviral based treatment of human disease. We have chosen leukocyte adhesion deficiency (LAD) as a model system for retroviral mediated gene transfer into human hematopoietic cells.13 LAD is a rare autosomal recessive disorder caused by a defective CD18 gene.14 The defective expression of CD18 leads to the decreased expression of a family of three heterodimeric proteins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mo1), and CD11c/CD18 (p150,95). Patients with LAD suffer a range of functional defects, and often succumb in childhood to recurrent bacterial infections.15 Bone marrow transplantation has proven curative, demonstrating that correction of the defect in hematopoietic stem cells is adequate to fully treat the disease.16 We (the authors) and other investigators have recently demonstrated that transduction with a CD18-encoding retrovirus can restore the expression of functional LFA-1 to lymphoblasts derived from a patient with LAD.17,18 Further, LAD clinical severity is linked to the level of CD18 expression, suggesting that even low levels of CD18 expression may produce substantial clinical improvement.15

Here we demonstrate the transfer and expression of a gene encoding murine CD18 in normal human bone marrow mononuclear cells grown in continuous perfusion LTBMC. Gene transfer was achieved with a simple 2-day infection using a high titer cell-free viral supernatant of resting, unfractoned bone marrow mononuclear cells. A significant number of CFU-GM colonies as well as LTC-IC were transduced with retrovirus, suggesting gene transfer to primitive hematopoietic cells. In addition, we demonstrated expression of murine CD18 in the transduced cultures at levels that if obtained in vivo, could be clinically significant.

MATERIALS AND METHODS

Generation of a high titer recombinant retrovirus encoding for murine CD18. The plasmid 17.419 which encodes for murine...
CD18 was cut with Xho I, and BamHI linkers were attached using standard subcloning procedures. The resulting 2,495-bp fragment was cloned into the BamHI site of pMFG, to yield the plasmid pM18. Both amphotropic and ecotropic retrovirus producer cell lines were generated by cotransfecting the plasmid pM18 and pSV2Neo into 9, cells or into cells by calcium/phosphate precipitation. Clones were screened for their ability to express murine CD18 on the cell surface of a CD11a cell line. The highest titer amphotropic clone (M18-2P28) was used to transduce U937 cells. The highest titer ecotropic clone was selected (M18-E13), and was used to cross-infect Q cri p cells five times to amplify retrovirus titer. The murine CD18 transduced Qdp cells were subcloned, and individual virus producing cells were isolated. The highest titer clone (15-2) was used to transduce bone marrow cells. A clone of the cross-infected producer cells (15-2) produced retrovirus of approximately 1 to 2 log higher titer than the conventional producer cell line (Fig 1B).

While an accurate limiting dilution titer is not possible with these viral vectors because they do not contain a selectable marker, comparison with similar vectors with a selectable marker would indicate that M18-IP28 had an approximate titer of 10^5 CFU/mL, and 15-2 had an approximate titer of 10^5 CFU/mL. All retrovirus producing clones were free of replication competent retrovirus as determined by a proviral rescue with 3T3 amplification assay.

U937 transduction. Exponentially growing U937 cells were cocultured with the amphotropic retrovirus M18-IP28 for 2 days and nonadherent cells were serially isolated to eliminate virus producing cells. Approximately 1 week after retrovirus transduction of U937 cells, they were analyzed by flow cytometry (see below) for murine CD18 expression. Flow cytometric analysis showed that less than 1% of the U937 cells expressed murine CD18. Transduced cells were FACs sorted twice for murine CD18 expression, and the resulting population U-M18-2 was used in subsequent experiments. Analysis by Southern blot of U-M18-2 cells demonstrated approximately one copy of unrearranged provirus per cell (data not shown).

U937 immunoprecipitation. Cell surface iodination and immunoprecipitation was performed essentially as described. Briefly, exponentially growing cells were iodinated by the lactoperoxidase method, solid phase immunoprecipitated with either an antihuman CD11a antibody (TS1/22), an antimurine CD18 antibody (M18), a control antibody for the antihuman CD11a antibody, or a control antibody for the antimurine CD18 antibody (all obtained from the American Type Culture Collection [Camden, NJ] and used as neat tissue culture supernatants), and were electrophoresed through an 8% nonreducing polyacrylamide gel, and were then subjected to autoradiography.

Flow cytometry. Flow cytometry was performed as described either without or with a 72-hour incubation in 48 phorbol 12-myristate 13-acetate (PMA) and representative histograms are depicted. Cells were subjected to indirect immunofluorescence with the following antibodies: TS1/18 (murine antihuman CD18), TS1/22 (mu-
M-CD18

H-CD18

H-CD11a

U937

0.1

13.4

11.7

U-M18-2

4.4

9.9

10.0

+ PMA

0.2

30.7

25.1

U-M18-2

14.2

32.8

30.1

Cell number

Immunofluorescence (log scale)

Fig 2. Murine CD18 can be expressed on the cell surface of human myeloid cell lines. Depicted are histograms of indirect immunofluorescence of untransduced cells (U937) or transduced and sorted cells (U-M18-2) either without or with a 72-hour incubation in PMA. Test antibody (anti-murine CD18-M18, antihuman CD18-H-CD18, or antihuman CD11a-HCD11a) is the solid line, control antibody is the dotted line. The specific fluorescence above control (linear scale) is shown in the upper right hand corner.
RESULTS

A recombinant retrovirus vector encoding murine CD18 was generated (Fig 1A). This vector transcribes predominantly from the retrovirus long terminal repeat. The inclusion of the endogenous envelope splice-accept sequences allowed for the generation of genomic provirus derived RNA suitable for the efficient translation of murine CD18. An amphotropic producer cell line was used for transduction of normal human bone marrow.

To confirm that murine CD18 could be expressed on the cell surface of myeloid cells, the macrophage-like cell line U937 was transduced with the amphotropic murine CD18 encoding retrovirus. As the initial transduction efficiency was less than 1% (data not shown), murine CD18⁺ cells were isolated by 2 sequential FACS sorts to generate the cell line U-M18-2 (Fig 2). Murine CD18 is efficiently expressed on the cell surface of these myeloid cells. Murine CD18 expression increases after incubation with the phorbol ester PMA, concomitant with an increase in the endogenous β2 integrin expression (Fig 2). These results suggest that the murine CD18 encoding vector can efficiently function in both resting and differentiated myeloid cells.

The β2 integrins require heterodimer formation to be efficiently expressed on the cell surface. Cell surface iodinated transduced cells (U-M18-2) were subjected to immunoprecipitation with antibodies directed at either murine CD18 or human CD11a (Fig 3). Immunoprecipitation of murine CD18 from the transduced cells confirms that murine CD18 is physically associated with human CD11a on the surface of the U937 cells. Precipitation with an antibody directed at human CD11a co-precipitated both murine CD18 and human CD18. These results provide supportive evidence for heterodimer formation between murine CD18 and human CD11a in human myeloid cell lines. This extends the results of others demonstrating heterodimer formation between murine CD18 and human CD11a in transfected fibroblasts and somatic cell hybrids.

Based on previous experiments using continuous perfu-

![Fig 3. Murine CD18 is in close proximity to human CD18 in transduced cells. U-M18-2 cells were iodinated by the lactoperoxidase method, solid phase immunoprecipitated with either an anti-human CD11a antibody (anti-H-CD11a), an antimurine CD18 antibody (anti-M-CD18), a control antibody for the anti-human CD11a antibody (CON-H) or a control antibody for the antihuman CD11a antibody (CON-M) and run on a 6.0% nonreducing polyacrylamide gel. Molecular weight markers in kilodalton are shown to the right.](image)

Table 1. Retrovirus Transduced Hematopoietic Cells Grown in Bioreactors Consistently Produce Provirus Positive Progenitors for 6 Weeks

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Cell Number, Cumulative, x 10⁶ (fold increase)</th>
<th>CFU-GM, cumulative (fold increase)</th>
<th>Provirus Positive CFU-GM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.0</td>
<td>18,600</td>
<td>14/14 100</td>
</tr>
<tr>
<td>1</td>
<td>5.1 (1.7)</td>
<td>40,100 (2.2)</td>
<td>13/13 100</td>
</tr>
<tr>
<td>2</td>
<td>11.2 (3.7)</td>
<td>90,915 (4.9)</td>
<td>8/12 57</td>
</tr>
<tr>
<td>4</td>
<td>28.1 (9.3)</td>
<td>119,580 (6.4)</td>
<td>9/10 90</td>
</tr>
<tr>
<td>6</td>
<td>35.8 (11.9)</td>
<td>141,680 (7.6)</td>
<td>6/12 50</td>
</tr>
</tbody>
</table>

*Cell number is the average of two bioreactors harvested at the indicated time points. Bioreactors run for more than 2 weeks were depopulated at 21 days by the removal of 75% of the nonadherent cells.
sion culture of hematopoietic cells, we postulated that rapid media exchange would stimulate hematopoietic cell replication and allow successful transduction with retroviral vectors. In order to investigate the ability of the murine CD18-encoding virus to infect early hematopoietic cells grown in rapidly perfused LTBMC, fresh normal human bone marrow mononuclear cells were inoculated into continuous perfusion culture chambers and the cultures were transduced with the murine CD18 encoding retrovirus I5-2 as described in Materials and Methods. Media was perfused through the chamber at 2.5 mL/24 hours, providing for a 50% volume exchange every 24 hours. Both the total cell number, and the total number of CFU-GM continuously increased for the entire 6-week period (Table 1). Similar to untransduced cultures, the maximum CFU-GM and overall cell number expansion occurred during the first 2 weeks of the long-term culture,
although continuous production of both CFU-GM was noted for the entire 6 weeks.

Analysis of DNA from isolated progenitor colonies demonstrated efficient gene transfer at all time points tested (Table 1 and Fig 4). PCR detection of proviral DNA revealed that provirus was present in a majority of CFU-GM colonies throughout the 6-week duration of the experiment. At the first analysis 1 week after infection, nearly all of the progenitor cell colonies contained proviral sequences. Continued analysis showed that the proportion of positive colonies never dropped below half of the colonies examined at any time point (Table 1 and Fig 4). Southern blot analysis with a viral-derived oligonucleotide probe internal to the PCR primers confirmed the identity of the PCR fragment (Fig 4D). As a control against the possibility that lysed cells could introduce free genomic DNA throughout the methylcellulose, resulting in false positives, cell-free areas of the progenitor cultures were also assayed by PCR, and were uniformly negative (data not shown).

To compare these data with that of a conventional LTBMC, we also cultured human marrow cells in conventional culture dishes as previously described. Cells were harvested from these cultures at 2 and 4 weeks of culture, and used in our CFU-GM assay. CFU-GM colonies derived from cells harvested at 14 days of culture were uniformly provirus negative when assayed by our PCR assay, and were uniformly negative (data not shown).

The presence of proviral DNA in CFU-GM colonies from day 42 of bioreactor culture led us to test how effectively earlier hematopoietic progenitor cells were infected with the retrovirus. LTC-IC represent cells hierarchically less mature than committed progenitors. Therefore, in a separate experiment, we examined CFU-GM derived colonies produced by 7-week LTC-IC culture as described previously. We reasoned that cycling of stem cells early in the culture would allow integration of provirus in cells measured in our LTC-IC assay. Using the same PCR assay as was used for CFU-GM provirus detection, we found that 33% (4 of 12) LTC-IC derived methylcellulose colonies from different cultures in our limiting dilution assay contained provirus (Fig 6). This suggests that at least a portion of the earliest cells in the new culture are cycling, which is in agreement with earlier work describing the expansion of these cells using this culture method.

Murine CD18 was efficiently expressed in a proportion of the transduced cells in culture. Flow cytometric analysis of both the nonadherent fraction as well as the adherent fraction of cells demonstrated expression of murine CD18 on the cell surface at both 4 weeks and 6 weeks (Fig 4B, C). The reduced murine CD18 expression in the adherent fraction may be due to the trypsinization required to harvest these cells. Murine CD18 fluorescence persisted at roughly steady levels throughout the 6-week duration of the experiment.

**DISCUSSION**

We have shown that a high titer retrovirus may be used as a vector for efficient gene transfer into human hematopoietic cells grown in continuous perfusion LTBMC, and primary hematopoietic cells can efficiently express murine CD18 on their cell surface. Immunoprecipitation of surface labeled cells suggests that murine CD18 is being expressed as a heterodimer with human CD11a. In addition, the retrovirus vector can upregulate murine CD18 protein expression after in vitro differentiation, suggesting that this vector can efficiently express protein in both early and mature hematopoietic cells. Genetic modification of 33% of LTC-IC suggests that a portion of the most immature cells are being transduced. The ability to expand and genetically modify normal hematopoietic cells may make it feasible to perform
a "competition transplant" with genetically modified bone marrow without significant myeloablation.

Hematopoietic stem cell gene transfer in animal systems has met with varied success. A number of investigators have achieved the long-term expression of transgenes in virtually all mice transplanted with genetically modified bone marrow. Reproducible transduction of the hematopoietic stem cell of mice has relied on the treatment of marrow donor mice with a cytotoxic agent such as 5-fluorouracil, the prestimulation of the donor marrow in vitro with a combination of early acting growth factors such as kit ligand (stem cell factor), interleukin 3 (IL-3), and IL-6, and the use of ecotropic murine retrovirus as the gene transfer substrates. Attempts to genetically modify the hematopoietic stem cells of mice with amphotropic retrovirus have generally resulted in overall lower gene transfer rates.

Genetic modification of the hematopoietic stem cell of larger animals, such as dogs and non-human primates has been less efficient. Even with a high-titer virus, and using repeated retrovirus transduction, fewer than 5% of hematopoietic progenitor cells can be reliably transduced. Similar work by others in primate stem cell gene transfer model has demonstrated roughly equivalent levels of gene transfer, with genetic modification occurring in at best 5% of the repopulating hematopoietic stem cells. In some instances persistence of the transgene has been observed for periods up to 1 year suggesting true stem cell genetic modification, albeit at relatively low rates. These results suggest that substantial improved gene transfer protocols need to be devised to obtain higher rates of gene transfer into large animals and humans.

Recently, several investigators have achieved relatively high rates of genetic modification of hematopoietic progenitors. Efficient genetic modification of up to 100% of CFU-GM has been obtained by transducing hematopoietic progenitors isolated from patients receiving human growth factor (HGF) isolated from patients during recovery from high-dose chemotherapy. In patients primed only with in vivo HGF, transduction of isolated CD34 cells in the presence of HGF resulted in 17% to 50% of the CFU-GM being genetically modified. Genetic modification of 63% of the CFU-GM was obtained by Moore et al, using resting marrow and allogeneic stromal cell feeder layers. Interestingly, in these experiments the transduction efficiency in the presence of bone marrow stromal cells was not appreciably enhanced by the supplementation with additional growth factors during the retrovirus transduction.

Hughes et al have reported the ability to transduce up to 60% of colony-forming cells (CFCs) and up to 40% of LTC-IC using a high titer neomycin encoding virus to transduce bone marrow collected during steady-state hematopoiesis. We have achieved virtually identical gene transfer rates, and in addition have demonstrated the cell surface expression of the transgene in a proportion of the transduced bone marrow cells. Although infection of cells that give rise to progenitor cells after 6 weeks of culture may reflect infection of the...
stem cell, human trials using these gene transfer techniques are needed to confirm transfer into stem cells rather than committed progenitor cells. (This level of CD18 expression, if obtainable in vivo in hematopoietic cells, may be of a level that could partially ameliorate the phenotype of leukocyte adhesion deficiency. Further experiments will be necessary to determine whether we can complement the defect in bone marrow derived from patients with LAD.

Continuously perfused culture vessels should continue to provide a useful model for gene therapy in the hematopoietic system for several reasons. This culture method appears to improve the efficiency of retroviral gene transfer when compared with conventional static cultures. A high serum turnover rate provides conditions analogous to in vivo bone marrow. Perfusion with HGF supplemented media can induce rapid cell cycling of both the hematopoietic and stromal elements, increasing the efficiency of retroviral gene transfer. A stromal bed can be established before marrow culture, thereby providing an optimal microenvironment for retroviral infection. It has been shown that extracellular matrix molecules improve the efficiency of retroviral gene transfer in hematopoietic cells. Culture in perfusion vessels has provided an important method for the efficient transduction of hematopoietic progenitors, while maintaining a significant expansion of myeloid progenitor cells.

The ability to achieve engraftment of normal marrow in mice hypertransfused with hematopoietic stem cells raises the possibility that a similar phenomena may occur in humans. If hematopoietic stem cells could be sufficiently expanded ex vivo, then presumably administration of a large number of ex vivo expanded and genetically modified hematopoietic cells could lead to the engraftment of a proportion of the genetically modified cells. Further improvements in both the transduction frequency and the ability to expand hematopoietic cells will be necessary before this hypothesis can be tested in the clinical setting.

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