Fetal Liver Hematopoietic Stem Cells As a Target for In Utero Retroviral Gene Transfer

By D. Wade Clapp, Luba L. Dumenco, Maria Hatzoglou, and Stanton L. Gerson

Retroviral-mediated gene transfer into hematopoietic precursors often results in only short-term gene transduction in vivo. Loss of the transduced genetic material over time may be caused by the limited ability of retroviral infection to transduce genes into early, pluripotent hematopoietic stem cells. Because fetal liver contains actively proliferating multipotent stem cells that should be more susceptible to retroviral-mediated gene transfer than quiescent cells derived from adult bone marrow, these cells may be an ideal target for gene transduction. Furthermore, physiologic expansion of these cells during development obviates the need for marrow ablation during gene therapy in vivo. We performed in utero gene transfer by injecting high titer replication-defective retrovirus in vivo into the livers of 11, 14, 16, and 18 day gestation rats. After birth, the rats were analyzed for the presence of proviral integration and gene expression. The efficiency of gene transfer into bone marrow cells was greatest in rats infected at day 14 to 16 of gestation. In rats killed at 1 to 26 weeks of age, gene transfer was detected by Southern analysis in 48% and by polymerase chain reaction in 86% of bone marrow samples. The provirus was also detected in white blood cells, the granulocyte-macrophage colony-forming unit, thymus, spleen, liver, and lung. The presence of the transgene in bone marrow and other hematopoietic tissues at 26 weeks of age suggests that early hematopoietic precursors present in the fetal liver are susceptible targets for gene transfer and that these cells become resident in the bone marrow of the adult animal. This model is a new technique for gene transduction into proliferating hematopoietic cells in vivo that avoids bone marrow transplantation and has potential application in the correction of genetic defects in utero.

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TRANSDUCTION OF FETAL HEMATOPOIETIC STEM CELLS

utero by intraperitoneal injection.\textsuperscript{21} Gene transfer and expression of the chimeric gene was demonstrated in the liver of rats analyzed up to 1 year of age.\textsuperscript{21} This study also suggested the feasibility of direct in utero retroviral transduction into hematopoietic cells because provirus containing the bovine growth hormone gene was identified in the BM of two rats killed at 3 months of age.\textsuperscript{21}

Because the fetal liver contains large numbers of hematopoietic precursors,\textsuperscript{15,16,22,23} we hypothesized that fetal liver injections with retrovirus would result in efficient gene transfer into multipotent and totipotent hematopoietic stem cells. These stem cells might then migrate from the fetal liver to the BM via the circulation,\textsuperscript{25-27} undergo physiologic expansion in the BM during early development, and result in efficient proliferation of hematopoietic cells containing the transgene. This approach might also avoid the use of lethal irradiation and BMT to achieve successful gene therapy.

MATERIALS AND METHODS

Animal Model

Artificially inseminated pregnant Sprague-Dawley rats (Zivic-Miller, Allison Park, PA) were used. In utero injection into the fetal liver was performed at day 11, 14, 16, or 18 of gestation by a modification of the technique described by Hatzoglou et al.\textsuperscript{21} Briefly, the pregnant rats were anesthetized, the abdomen was steriley prepped, and a midline abdominal incision performed. The uterine horns were externalized and the fetuses were oriented within the amniotic sac such that direct visualization and injection of the fetal liver could be accomplished. Twenty-five microliters (day 11 of gestation), 50 \(\mu\)L (day 14), or 100 \(\mu\)L (day 16 and 18) of retrovirus was injected into the liver of each fetus. The uterine horns were then returned to the abdomen, the maternal peritoneum closed in two layers, and the fetuses were allowed to complete gestation and to deliver normally.

Chemicals and Reagents

Tissue culture reagents were obtained from K.C. Biological Inc (Lenexa, KS). Calf serum was from HyClone Laboratories (Logan, UT). Hemin was obtained from Eastman Kodak (Rochester, NY). Pokeweed mitogen and genetin (G418) were obtained from Gibco (Grand Island, NY). Gene Screen Plus and \(^{38}\)P-dCTP were purchased from New England Nuclear Corp (Boston, MA). Ag-50-XA8 resin was obtained from BioRad (Richmond, CA). Taq polymerase and the reagents for DNA amplification were purchased from Perkin Elmer Cetus (Norwalk, CT) and used as recommended by the manufacturer. All other molecular biology reagents were obtained from Boehringer Mannheim (Indianapolis, IN) and used as suggested by the manufacturer. General laboratory chemicals were from Sigma (St Louis, MO).

Retroviral Vector

The retroviral vector pLJR8Vada\textsuperscript{28} was derived from pLJ (provided by R. Mulligan, Whitehead Institute, MIT) and contains the bacterial neomycin resistance gene neo under the transcriptional control of the viral long terminal repeat (LTR), and the Escherichia coli DNA repair gene ada, which produces the O'alkylguanine-DNA alkyltransferase protein (Fig 1). In pLJR8Vada, the ada gene is under the transcriptional control of the 621-bp fragment of the Rous Sarcoma Virus (RSV) 3' LTR used as the internal promoter.\textsuperscript{27} Virus produced by \(\psi\)-2 cells\textsuperscript{29} transfected with pLJR8-
rat spleen cell-conditioned media prepared as previously described.\textsuperscript{26} 30\% vol/vol fetal calf serum, and 10\% vol/vol Iscove's modified Dulbecco's medium. Mononuclear cells were plated at 10^6/mL. The cultures were incubated at 37°C, 5\% CO_2 for 7 to 10 days. Colonies greater than 50 cells were counted and colonies greater than 200 cells were aspirated for analysis of gene transfer (see below).

**Nucleic Acid Analysis**

*Southern analysis.* Cellular genomic DNA was prepared as previously described.\textsuperscript{26,29} Southern analysis was performed by digesting 20 \mu g samples of genomic DNA with either Xba I or Kpn I. Digested DNA was separated by electrophoresis on a 1\% agarose gel. The probes were used were the 1.2-kb neo gene and the 1.3-kb ada gene labeled with \textsuperscript{[32P]} dCTP with random oligonucleotide primers. Hybridization was performed at 42°C for 48 hours as described.\textsuperscript{26,28} Filters were washed with 0.1\% sodium dodecyl sulfate (SDS), 0.1X SSC at 55°C, dried, and exposed to Kodak XAR film at -80°C. The expected fragments of genomic DNA derived from cells infected with vLJRS\textsubscript{ada} and hybridizing with the ada probe were 1.9 kb following Xba I digestion and 5.7 kb following Kpn I digestion (see Fig 1). Genomic DNA isolated from cells infected with vLJad\textsubscript{a} and digested with Xba I or Kpn I should generate fragments of 3.8 kb that hybridize with the ada or neo probes.

*Functional analysis.* Twenty micrograms of genomic DNA from vLJad\textsubscript{a} was digested with Cla I that recognizes a unique site within the provirus 3' to the ada gene (Fig 1). Digested DNA was then separated by agarose gel electrophoresis, prepared for Southern analysis as above, and hybridized with the \textsuperscript{[32P]}-labeled ada probe.

**PCR**

Two micrograms of genomic DNA isolated from various tissues was used as substrate for detection of small amounts of the transduced ada gene by use of the PCR as previously described.\textsuperscript{11} Laboratory procedures were used that avoid contamination of genomic DNA with the PCR product or with proviral sequences. DNA from uninfected rats was run as a control in each experiment. Thirty picomoles of two 21-bp oligonucleotides were used in a standard PCR reaction. The PCR generated a 633-bp fragment of genomic DNA was subject to limiting dilution before PCR analysis. Thus, it appears that the in utero injection procedure rather than the retrovirus itself has associated fetal mortality.

**Gestational Age-Dependent Gene Transfer**

To define whether gene transfer into hematopoietic cells was affected by the gestational age at the time of retroviral infection, rats injected at 11 to 18 days of gestation were killed at 1, 3, and 8 weeks postnatal age and analyzed for the presence of proviral sequences in genomic DNA from liver, BM, spleen and lung. Samples were evaluated for the presence of provirus in genomic DNA by the sensitive PCR method and analyzed for the efficiency of gene transfer by Southern analysis of restriction enzyme-digested genomic DNA from uninfected rats such that each pregnant rat was injected with 100 \mu L media alone was similar to those injected with 100 \mu L retrovirus supernatant (16 of 26 in the retrovirus group and 10 of 15 in the media group). Thus, it appears that the in utero injection procedure rather than the retrovirus itself has associated fetal mortality.

**RESULTS**

**Fetal Retroviral Injection and Survival**

Fetal rat liver was injected in utero with replication-defective retrovirus at day 11, 14, 16, or 18 of gestation with, depending on the age, between 5 \times 10^6 to 5 \times 10^7 infectious retrovirus/fetus. There was a significant in utero fetal mortality associated with the in utero injections, especially during the initial experiments. However, all animals alive at birth developed normally. The mean fetal survival in rats who were injected with concentrated retrovirus was 32\% (Table 1). Fetuses that were injected at day 11 gestation had a significantly lower (17\%) rate of survival than did fetuses injected at days 14, 16, or 18 of gestation (mean, 37\%). In a second group of experiments, survival of day 16 gestation fetuses that received injections of 100 \mu L media alone was similar to those injected with 100 \mu L retrovirus supernatant (16 of 26 in the retrovirus group and 10 of 15 in the media group). Thus, it appears that the in utero injection procedure rather than the retrovirus itself has associated fetal mortality.

**Table 1. Fetal Survival Following Injection of Concentrated Retrovirus**

<table>
<thead>
<tr>
<th>Pregnant Rate per Group</th>
<th>Gestational Age at Injection</th>
<th>Injected (N)</th>
<th>Liveborn (N)</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>11</td>
<td>47</td>
<td>8</td>
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<td>40</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>52</td>
<td>20</td>
<td>38</td>
</tr>
</tbody>
</table>

Pregnant Sprague-Dawley rats at 11, 14, 16, or 18 days of pregnancy were anesthetized using ether. The uterine horns were externalized. The liver of each fetus was injected with concentrated retrovirus supernatant using volumes of 25, 50, 100, or 100 \mu L for fetuses of 11, 14, 16, or 18 days of gestation, respectively. The uterine horns were returned to the abdomen and gestation was allowed to continue to term, which is 22 days. Fetal survival was determined within 24 hours of birth.
DNA. Figure 2A shows a representative Southern analysis of genomic DNA. Fig 2B shows PCR analysis of tissues of four rats, and Table 2 shows the composite results of gene transduction by both PCR and Southern analysis. Overall, in BM the provirus was detected in 5 of 8 rats injected at day 11 of gestation, 10 of 12 rats injected at day 14 to 16, and 4 of 14 rats injected at day 18. In rats injected at day 14 to 16 of gestation, 7 of 10 BM samples with provirus detected by PCR also had provirus detected by Southern analysis, whereas 0 of 22 rats injected at 11 or 18 days of gestation had detectable provirus seen on Southern analysis. Because PCR may detect very few cells (< 1%) carrying the provirus while detection by Southern analysis of genomic DNA requires about 10% of cells to carry the provirus, these results suggest much more efficient gene transfer in rats injected at day 14 to 16 of gestation than at earlier or later times.

Gene transduction was also detected in other tissues of animals injected at day 14 to 16 of gestation but at a lower

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**Table 2. Tissue-Specific Gene Transfer**

<table>
<thead>
<tr>
<th>Day of Gestation (Injected)</th>
<th>Animals Tested</th>
<th>Any Organ</th>
<th>BM</th>
<th>Liver</th>
<th>Spleen</th>
<th>Lung</th>
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<tbody>
<tr>
<td>11</td>
<td>8</td>
<td>5</td>
<td>5*</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>14</td>
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<td>4</td>
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<td>4</td>
<td>3</td>
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<td>14</td>
<td>5</td>
<td>4*</td>
<td>1*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Animals were killed at 1 to 8 weeks of age and evaluated for the presence of provirus by Southern analysis and PCR. For Southern analysis, genomic DNA was digested with Kpn I or Xba I, separated by agarose gel electrophoresis, and transformed to a nylon membrane followed by hybridization with [32P] ade. Twenty-two of 34 animals had evidence of gene transfer.

*Gene transfer for this group was detected only by PCR. For other groups, some tissues had gene transfer detected by PCR and others by Southern analysis.*
frequency than in the BM. Liver was positive by PCR in 7 of 12 rats (4 of 12 by Southern analysis), spleen in 8 of 12 rats (4 of 12 by Southern analysis), and lung in 6 of 12 rats (3 of 12 by Southern analysis). In contrast, proviral sequences were detected in organs other than BM in only 1 of 14 rats injected at day 18 gestation and in none of the other organs of rats injected at day 11 gestation. No correlation was noted between the estimate of gene copy number and the age of the animal at the time of harvest. In most instances, intact proviral sequences were found when the blots were probed with [32P] ada or neo fragments; however, one liver sample showed a small deletion.

**Long-Term Persistence of Retrovirus After In Utero Injection**

In a separate set of experiments, we determined whether the in utero method of gene transfer could achieve successful long-term gene transduction into one or more hematopoietic cell lineages. Sixteen of 26 fetuses from three pregnant rats injected with vLJuda retrovirus at day 16 of gestation survived and delivered at term. Nine rats were killed at 26 weeks of age for analysis of gene transfer.

To evaluate the presence and persistence of the provirus in a single group of rats over time, white blood cells (WBCs) were isolated from heparinized blood, collected at 4, 18, and 26 weeks of age, and were analyzed by PCR. The provirus was detected by PCR in 6 of 12 animals at 4 weeks of age, 2 of 11 animals at 18 weeks of age, and 6 of 9 animals at postmortem exam at 26 weeks. We may have underestimated the proportion of rats with provirus in their blood cells based on a recent report describing the ability of heparin to interfere with the PCR reaction.32

From rats killed at 26 weeks of age, BM, thymus, blood, spleen, liver, and lung were harvested for genomic DNA and analyzed for gene transduction by PCR and by Southern analysis (Table 3). Eight of nine animals contained the provirus in the BM by PCR. Of these, three of nine were detected by Southern analysis of the BM at an estimated efficiency of the three rats of between 10% and 90% of the cells carrying the provirus. In one of three of these rats, gene transduction was found in WBCs as well. In four other rats that had provirus detected by PCR in blood cells, provirus was also detected in BM by PCR. The provirus was also detected by PCR in most rats injected with retrovirus in the thymus, spleen, liver, and lung, and to a lesser extent by Southern analysis of genomic DNA.

Figure 3 presents a detailed analysis of two rats that contained evidence of provirus in multiple organs at 26 weeks. In rat 16-1 (Fig 3A) provirus was detected by Southern analysis of genomic DNA in BM, liver, and lung. Junctional analysis (Fig 3Ab) to detect an oligoclonal pattern of cell progeny showed one or two bands per tissue with an apparently identical band in liver and BM, suggesting a clonal origin to the cells (perhaps a monocyte precursor). Spleen and thymus, which did not contain provirus detectable by Southern, did have provirus detected by PCR (Fig 3Ac). In rat 16-2 (Fig 3B) provirus was detected in thymus, liver, lung, and spleen by Southern analysis whereas junctional analysis showed two prominent bands in the thymus but no bands in other tissues. BM provirus was not seen by Southern analysis but was seen by PCR analysis to a 1:300 dilution (Fig 3Bc). In contrast, PCR analysis of the BM from rat 16-1, which was detected by Southern analysis, detected provirus to a 1:3000 dilution. Thus, rat 16-2 has evidence of a clonal pattern in lymphoid cells but had a relatively low level of gene transduction into BM cells, precluding an analysis of the clonal pattern in these cells. Analysis of expression of the transduced ada gene failed to detect ada mRNA by Northern analysis of total cellular RNA from liver or BM. In addition, no ada derived alkyltransferase protein was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown).

We next asked whether hematopoietic progenitors from in utero infected animals contained the provirus. BM cells were cultured for CFU-GM in vitro from five animals killed at 26 weeks. Individual progenitors were harvested on day 7 of culture. DNA was isolated and amplified using PCR. A total of 23 CFU-GM colonies were examined. Twelve of 23 CFU-GM contained the provirus (Fig 4). Each of the four rats examined that contained the provirus in the BM had provirus detected in CFU-GM.

**DISCUSSION**

We have found that in utero retroviral infection is a promising means of gene transfer into hematopoietic precursors in vivo. When in utero fetal liver injections were performed between day 14 and 16 of gestation, detectable proviral sequences were observed in live-born rats and these sequences persisted until the rats were killed at 26 weeks of age. In our initial experiments, 10 of 12 rats injected at day 14 to 16 of gestation had evidence of gene transfer into hematopoietic cells, and in the second set of
Fig 3. Analysis of gene transfer at 26 weeks of age. Two rats, 16-1 (A) and 16-2 (B) were injected with vLJad at day 16 of gestation and harvested at 26 weeks of age. Southern (a), junctional fragment (b), and PCR analyses (c) were performed on tissues from each animal. (a) Southern analysis: Twenty micrograms of genomic DNA was digested with Kpn I separated on a 1% agarose gel and hybridized with [32P]-labeled ada. Expected fragment size is 3.8 kb. (b) Junctional analysis: Genomic DNA was digested with Cla I, which cuts once in the provirus and Southern analysis performed as in (a). Each band marks a prominent clone of cells containing the provirus. (c) PCR analysis: Tissues negative by Southern analysis were analyzed for efficiency of gene transfer by PCR. For each tissue, serial dilutions of 2 μg genomic DNA from infected rats was mixed with genomic DNA from the same tissue of an uninfected rat to yield a total of 2 μg DNA and the mixture used as substrate in the PCR incubation. PCR analysis of rat 16-1 BM is shown for comparison.

experiments, 8 of 9 rats injected at day 16 of gestation had provirus detected in the BM at 26 weeks of age.

Persistence of provirus in peripheral blood and BM cells as well as in thymus and spleen of 26-week-old rats suggests that early hematopoietic progenitors had been transduced. Analysis of unique insertion sites of the provirus (junctional fragment analysis) in two rats identified an oligoclonal pattern of transduced cells, suggesting that only a few stem cells had been infected or were supporting hematopoiesis at 26 weeks of age. In the clonal analysis of one rat, the BM and liver had one band of identical size, indicating the presence of cells (perhaps macrophages) derived from the same clone in the two organs. Because provirus was detected in both the thymus, spleen, and BM of five separate rats by PCR (Table 3), it is possible that a true totipotent stem cell has been infected in these animals. Unfortunately, this could not be confirmed by junctional analysis because the level of gene transfer was too low to consistently be identified by Southern analysis of genomic DNA. Alternatively we may have transduced several committed hematopoietic stem cells of either lymphoid or myeloid lineages. Thus, while it is clear that this technique has the potential for gene transfer into early pluripotent progenitors, further experiments are required to define the frequency of this occurrence.

Because the fetus is rapidly growing during midgestation, we hypothesized that proliferating totipotent stem cells would be susceptible to gene transfer in utero. We chose to study the period between 11 and 18 days of gestation because in rodents hematopoietic cells shift from the yolk sac to the fetal liver at about day 10 of gestation.33,34 Hematopoiesis subsequently expands in the liver such that by day 14 to 16 of gestation the fetal liver contains large numbers of hematopoietic stem cells that make up as much as 0.2% of total cells.35 At day 15 of gestation in the rat, nearly 90% of all CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) are located in the fetal liver.36 At approximately day 18 of gestation, the number of hematopoietic cells in the liver decreases37,38 as the BM cavity matures and hematopoiesis begins in the BM where it

Fig 4. PCR analysis of CFU-GM. BM of rats injected with vLJad at day 16 of gestation and harvested at 26 weeks of age was cultured for CFU-GM, individual colonies harvested, and DNA was extracted and amplified by PCR, as described in Materials and Methods. The product was separated by electrophoresis on a 2% agarose gel, transferred to Gene Screen Plus, and probed with a [32P]-labeled ada fragment. Brackets indicate CFU-GM from individual rats.

Total 12/23 CFU-GM Contain Provirus
is well developed by about day 20. On the basis of these developmental studies, our finding of proviral sequences in the BM of animals transduced in utero by retroviral injection into the liver provides indirect evidence for the migration of hematopoietic stem cells from the liver to the BM. These studies also may explain why the maximum efficiency of gene transfer to BM occurred in animals injected at day 14 to 16 of gestation; at this age the fetal liver contains the greatest proportion of hematopoietic precursors, whereas the BM cavity is still immature and contains few hematopoietic precursors.\(^5\)

Three issues need to be addressed to optimize this technique of gene transfer: (1) improving the efficiency of retroviral gene transfer; (2) reducing the mortality of the in utero injection; and (3) using vectors that will allow gene expression in hematopoietic cells. The efficiency of gene transfer to BM is related to the number of viral particles injected and the proliferative capacity of the hematopoietic cells infected. Because we injected only \(5 \times 10^4\) to \(5 \times 10^6\) particles into each fetus, we rely on the rapid proliferation of hematopoietic stem cells in the fetus (which is age-dependent) to amplify the number of cells that contain the transgene. Higher virus titer may improve the level of gene transfer that takes place.

The second issue, fetal mortality, was a significant problem in our study at each gestational age tested. Fetal demise appeared in large part to be due to the volume injected rather than the presence of retrovirus because it was similar in fetuses injected with either media or retrovirus. Causes could include hemorrhage from needle puncture, abortion secondary to fetal manipulation, or adverse effects of increased pressure due to the volume of fluid injected. Improved techniques of injection and the use of smaller volumes of retroviral supernatant containing higher virus titer may decrease the fetal mortality associated with this procedure.

Third, we have not detected expression of the transduced genes in any of the tissues examined. Northern analysis of total cellular RNA from multiple organs failed to identify a proper transcript for either the \(ada\) or neo gene when probed with either a standard DNA probe or a riboprobe.\(^3,36,37\) Further, no bacterial O\(\beta\)alkylguanine-DNA alkyltransferase derived from the \(ada\) gene was detected by SDS-PAGE.\(^1\) CFU-GM colonies from adult rats infected in utero did not show drug resistance to G418. This lack of expression may indicate that the pLJ-based construct is not optimal for gene expression in hematopoietic cells. We have also been unsuccessful demonstrating expression of vLJRSV\(v\)ada in mouse CFU-S (data not shown), even though we have shown that the \(ada\) and neo genes are expressed in fibroblast cell lines and that transcripts derived from the 5' LTR and the internal RSV promoter are present in vitro.\(^2\) Other retroviral constructs may be more likely to express internal genes in hematopoietic cells in vivo.\(^3,36,38\)

We also observed that nonhematopoietic tissues, specifically liver and lung, contained low levels of transduced provirus. In most instances, the proportion of cells containing the provirus was small, typically less than 5%, and could be detected only by PCR. Our results are similar to those observed by Hatzoglou et al,\(^1\) who identified proviral sequences in a variety of tissues after fetal liver injections. Our studies were designed to target hematopoietic cells, whereas the earlier study was aimed at expressing genes in the liver by performing injections later in development (day 19). Both studies have shown that retroviral-mediated gene transduction in utero can yield long-term, stable gene transfer but that it is not a tissue-specific or restricted process.

In summary, in utero retroviral infection of fetal liver leads to stable gene transduction for at least 6 months of BM hematopoietic progenitors of the developing rat. This finding strongly suggests that early hematopoietic stem cells have been infected without the need to resort to either BM ablation followed by transplantation\(^1\) or special strains of mice with genetic defects in hematopoiesis.\(^30,40\) This model could have application in the analysis of gene transduction in larger animals, and has potential clinical application in the correction of human genetic diseases.

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