Retroviral Vector-Mediated Transfer of the Bacterial Neomycin Resistance Gene Into Fetal and Adult Sheep and Human Hematopoietic Progenitors In Vitro

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We compared the efficiency of retroviral vector (N2)-mediated transfer of the bacterial neomycin resistance gene (Neo^R) into adult and fetal hematopoietic progenitors of sheep and humans by assessing their ability to form colonies in the presence of lethal doses of the neomycin analogue G418 in vitro. Fetal cells from both sheep and humans exhibited a higher degree of Neo^R transfer than adult cells. The overall level of Neo^R expression was significantly higher for sheep than human cells. The transfer/expression of Neo^R into adult human bone marrow hematopoietic progenitors was not affected by the presence or absence of T cells and monocyte/macrophages. The efficiency of Neo^R transfer into both adult and fetal human cells, however, was improved when transduction was carried out in the presence of recombinant human interleukin-3 and granulocyte-macrophage colony-stimulating factor. These results demonstrate the greater efficiency of Neo^R gene transfer into fetal hematopoietic progenitors, which may provide a basis for the relatively higher efficiency of the in utero approach to gene therapy.

GENE THERAPY INVOLVING the transfer of a functioning exogenous gene into the appropriate somatic cells (eg, hematopoietic cells) of an organism offers a precise means of treating a genetic disease.[1-4] The insertion of genes into hematopoietic cells has been greatly facilitated by the use of retroviral vectors.[1-9] Genes can be transferred with high efficiency into mouse hematopoietic cells[10-12] and rabbit endothelial cells,[13] and, with lesser efficiency, into dog[14] and human[6,8,15] hematopoietic cells. Postnatal gene transfer studies in dogs[14] and monkeys[9] using a bone marrow transplantation/retroviral gene transfer protocol demonstrated the feasibility of gene transfer and expression in large animals. However, the transfer/expression occurred at very low efficiency.[9-14] Using an in utero gene transfer protocol involving the retroviral-mediated transfer of the bacterial neomycin resistance gene (Neo^R) into hematopoietic cells obtained from the circulation of fetal sheep, we recently reported the long term expression of the Neo^R gene in sheep at significantly higher efficiency than has been observed in other large animal models.[16] In the present study, we explored the possibility that the relatively higher level of Neo^R transfer/expression found in sheep using the in utero gene transfer protocol correlates with a greater efficiency of retroviral-mediated Neo^R transfer into fetal than into adult hematopoietic progenitors. The results presented here demonstrate that fetal hematopoietic progenitors from both sheep and humans exhibit significantly greater efficiency of retroviral-mediated Neo^R transfer than adult hematopoietic progenitors, and the efficiency of Neo^R transfer was higher in sheep than in human cells.

MATERIALS AND METHODS

Preparation of adult sheep and human bone marrow. Bone marrow was aspirated from the posterior iliac crest, and placed into heparinized (preservative-free) Iscove's Modified Dulbecco's Medium (IMDM). The samples were centrifuged at 600 × *g* for 15 minutes, and the buffy coat cells were separated, washed in IMDM, 2% fetal calf serum (FCS) twice and resuspended in IMDM, 10% FCS. Bone marrow was obtained from normal, healthy adult volunteer donors after obtaining informed consent. Bone marrow mononuclear cells (BMC) were isolated by Ficoll-Hypaque density-gradient centrifugation (1.077 sp. gravity). When required, BMC were depleted of monocytes (MO) by adherence to plastic surfaces, and of T cells by rosetting with sheep red blood cells.[17]

Preparation of sheep and human fetal liver cells. Livers were obtained from sheep fetuses delivered by Caesarian section at 90 to 100 days of gestation. These fetuses also served as the source of fetal sheep bone marrow cells. Fetal livers were obtained from aborted human fetuses (17 to 23 weeks of gestation). After women had independently requested and scheduled pregnancy termination, informed consent was obtained for the use of the tissue for research. In order to prepare fetal liver cells, slices of liver were washed (three times in heparinized IMDM, 10% FCS) and passed through a sterile wet stainless steel screen mesh (0.2 mm pore size) under constant stream of IMDM, 10% FCS. Single cell suspension was obtained by passage of the mixture through a 25-gauge needle. The mixture was allowed to stand at room temperature for 5 minutes and the top 2/3 of the cell suspension was removed, pelleted by centrifugation (600 x *g*, 10 minutes), and in the case of fetal sheep liver, resuspended in IMDM, 10% FCS and kept at 4°C until used. Human fetal liver hematopoietic cells were further processed by isolation on Ficoll-Hypaque density gradient prior to use.

Preparation of fetal sheep bone marrow. Single cell suspensions of fetal sheep bone marrow were prepared by flushing femurs obtained from 90- to 100-day-old fetuses (see above) with heparinized IMDM, 10% FCS. The cell suspension was passed through a 25-gauge needle, washed twice, and resuspended in IMDM, 10% FCS.

Gene transfer protocol. The N2 vector, a Moloney murine leukemia virus-based vector with the viral coding sequences removed and the Neo^R gene from the bacterial Tn5 transposon inserted into it,

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was used in these studies. The construction and packaging of this vector in PA317 cells is described in detail elsewhere.[18,19] The virus-containing medium (VCM) had a titer for Neo^R of 1.6 × 10^6/mL on NIH3T3 cells and was minimally (less than 10%) contaminated with amphotropic helper. The following procedure was used to transduce the hematopoietic cells of sheep and humans. VCM was diluted 1:3 for sheep experiments and 1:5 for human experiments with fresh IMDM, 10% FCS, and polybrene was added to a final concentration of 8 ug/mL. Sheep adult and fetal cells were added at a concentration of 2.5 x 10^5 cells/mL to 40 mL of diluted VCM/polybrene mixture, mixed thoroughly and incubated overnight (12 to 14 hours) at 37°C (5% CO_2 in humidified air) with frequent gentle agitation. The cells were then pelleted, washed twice, and resuspended in IMDM, 10% FCS. Human adult and fetal cells were added at the same concentration to diluted VCM/polybrene mixture in the presence or absence of 100 ng/mL recombinant human interleukin-3 (IL-3, Genetics Institute, Cambridge, MA) and 400 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Genetics Institute) (with adult bone marrow cells) or 2.5 ng/mL IL-3 and 50 ng/mL GM-CSF (with fetal liver cells), mixed gently about every hour while incubating for 20 to 24 hours at 37°C (5% CO_2 in humidified air). The cells were then pelleted, washed and resuspended in IMDM, 10% FCS.

Hematopoietic progenitors assays. The presence/activity of the Neo^R gene in hematopoietic progenitors was assayed by testing adult and fetal hematopoietic cells for resistance to the neomycin-like antibiotic G418 (GIBCO, Grand Island, NY). Aliquots of adult sheep bone marrow cells (2 to 8 × 10^5/mL), sheep fetal marrow and liver cells (0.4 to 2 × 10^5/mL), human bone marrow cells (2 to 4 × 10^5/mL), human bone marrow cells depleted of MO and T cells (0.5 to 4 × 10^5/mL), and human fetal liver cells (4 × 10^5/mL) were cultured in plasma clot (colony forming unit-erythroid [CFU-E], burst-forming unit-erythroid [BFU-E]) or methylcellulose (CFU-Mix, CFU-C, BFU-E) as described.[16,20] The cells were cultured in the presence or absence of different concentrations of G418 (0.1 to 2 mg/mL), erythropoietin (0.4 IU/mL with sheep cells, 0.5 IU/mL with human cells), and a preparation of phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM) (5% vol/vol) derived from sheep or human blood leukocytes. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 4 (sheep CFU-E), 7 (human CFU-E), 9 to 12 (sheep CFU-Mix, CFU-GM, BFU-E), and 14 (human CFU-Mix, CFU-GM, BFU-E) days. Colonies in plasma clot were examined after transfer, fixing, and staining on glass slides; colonies in methylcellulose cultures were enumerated in situ.

RESULTS

In a series of studies, the effect of different concentrations (0.1, 0.4, 1.2, 1.5, and 2.0 mg/mL) of G418 on colony formation by normal (untransduced) fetal and adult sheep and human hematopoietic progenitors in vitro was determined. Figure 1 shows that the formation of colonies by normal sheep and human fetal and adult CFU-Mix, CFU-GM, and BFU-E was inhibited by G418 in a dose-dependent fashion. Some colonies were detectable at 1.5 mg/mL G418. At 2 mg/mL, however, no colonies were observed with human cells, and sheep cells produced very few colonies. In subsequent experiments, therefore, a concentration of 2 mg/mL G418 was used.

Results presented in Table 1 demonstrate the relative efficiency of Neo^R transfer into sheep hematopoietic progenitors in vitro. When compared with untransduced cells, colony

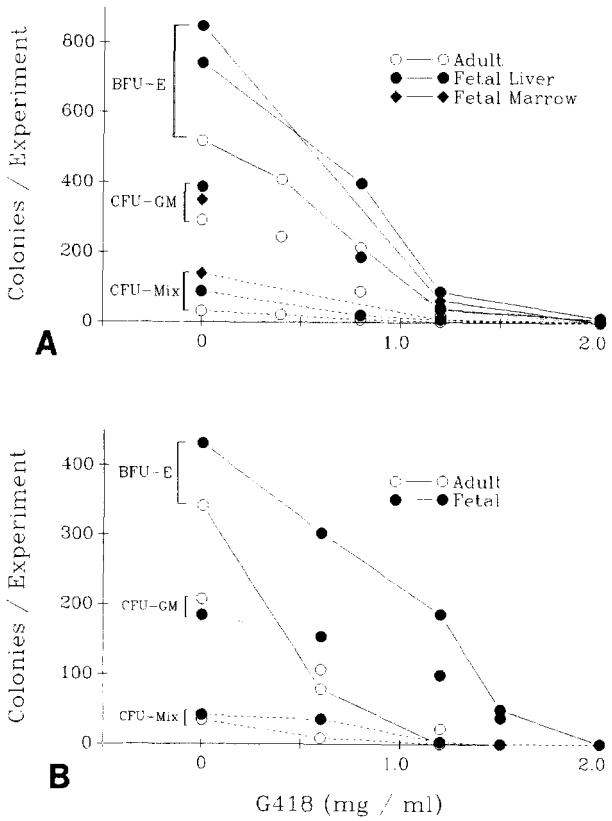

Fig 1. Effect of G418 on colony formation by normal sheep (A; each value represents mean of results from five different normal sheep) and human (B; data from a single experiment is presented; similar results were obtained in four additional studies) fetal and adult hematopoietic progenitors in vitro.

growth by vector-treated cells was normal in number, morphology, and size in the absence of G418 selection. At 2 mg/mL of G418, a small number of colonies were observed in cultures of untreated cells. By contrast, significant numbers of colonies produced by all four progenitor classes assayed were detected in cultures of transduced cells in the presence of 2 mg/mL G418 (Table 1). For a majority of these progenitors, those derived from fetal sheep exhibited greater efficiency of Neo^R transfer than adult cells. However, the differences between fetal and adult sheep erythroid progenitors were not as pronounced. Vector-exposed fetal BFU-E were only moderately more resistant to G418 than adult sheep marrow BFU-E, and there was essentially no difference in the numbers of G418-resistant CFU-E (Table 1).

The results of studies with human hematopoietic cells are presented in Table 2. As with the sheep, N2-treated human cells cultured in the absence of G418 produced hematopoietic colonies that were normal in number, size, and morphology, indicating that the gene transfer protocol used in these studies did not adversely affect these progenitors. Unlike the sheep, however, we did not detect any G418-resistant CFU-Mix in cultures of infected adult human bone marrow cells (Table 2). However, human fetal liver cells exposed to the N2 vector exhibited about 5% G418-resistant CFU-Mix–

Table 1. Efficiency of Neo^R Transfer Into Adult and Fetal Sheep Hematopoietic Progenitors In Vitro

	Total No. Colonies Enumerated*							
	No G418				2 mg/mL G418			
Cells Cultured	CFU-Mix	CFU-GM	BFU-E	CFU-E	CFU-Mix	CFU-GM	BFU-E	CFU-E
Adult Marrow								
Control	128	1,168	2,076	8,240	0 (0)	0 (0)	32 (1.5)	56 (0.7)
Transduced	160	1,272	1,952	7,384	19 (11.9)	88 (6.9)	300 (15.4)	1,072 (14.5)
Fetal Liver								
Control	356	1,548	2,968	23,544	0 (0)	0 (0)	52 (1.8)	0 (0)
Transduced	288	1,848	2,720	25,556	72 (25.0)	364 (19.7)	672 (24.7)	3,956 (15.5)
Fetal Marrow								
Control	556	1,396	3,576	46,400	0 (0)	16 (1.2)	0 (0)	76 (0.2)
Transduced	504	1,616	2,876	11,600	108 (21.4)	336 (20.8)	600 (20.9)	1,728 (14.9)

Four separate experiments, each involving a separate fetal and adult donor, were conducted. Each cell preparation was cultured at 2 to 8 $\times$ 10^5 cells/mL (adult) or 0.4 to 2 $\times$ 10^5 cells/mL (fetal) as described.

*Each value represents the total number of colonies assessed in the four separate experiments. Values in parentheses indicate percent resistant colonies.

derived colonies in vitro. Human fetal liver cells also produced higher percentages of G418-resistant CFU-GM colonies than adult human marrow cells, and unlike the sheep, infected human fetal liver cells exhibited higher percentages of G418-resistant BFU-E and CFU-E than human adult marrow (Table 2).

The results presented in Table 3 demonstrate that transduction in the presence of IL-3 and GM-CSF improved the efficiency of Neo^R transfer into both human adult and fetal hematopoietic progenitors. However, the level of improvement varied among the different progenitor classes, which may reflect the differential effect of these growth factors on these progenitors.

To determine whether the efficiency of transduction can be improved by increasing the number of progenitors exposed to VCM, the efficiency of transduction of whole bone marrow cells was compared with bone marrow cells depleted of MO and T cells. BMC (5×10^6) or BMC-MO-T were exposed to VCM (see Materials and Methods) and then cultured in methylcellulose and plasma clot in the presence or absence of 2 mg/mL G418. Table 4 shows that there was no improvement in the efficiency of Neo^R transfer when T- and MO-depleted BMC was used, despite the increased progenitor incidence in culture.

DISCUSSION

The results presented here demonstrate the greater efficiency of retroviral-mediated Neo^R transfer into fetal hematopoietic progenitors than into adult progenitors in vitro. This was apparent from the greater percentages of fetal progenitors of nearly all classes that exhibited resistance to G418 after exposure to the retroviral vector. This difference was more pronounced for human than for sheep cells. However, the efficiency of Neo^R transfer was considerably higher in sheep than in human cells. The average overall percentage of G418-resistant progenitors for adult sheep was 12.20 $\pm$ 1.90%, as compared with 0.85 $\pm$ 0.33% for adult human bone marrow progenitors. Similarly, 21.22 $\pm$ 2.27% of fetal sheep liver (and 19.49 $\pm$ 1.54% of fetal sheep marrow) hematopoietic progenitors exhibited G418-resistance, while 5.33 $\pm$ 0.36% of human fetal progenitors survived exposure to 2 mg/mL G418. It is unlikely that these differences in the efficiency of Neo^R transfer between sheep and human cells were associated with the different ratios of viral particles to target cells used in these studies. Although the ratio of viral particles to target cells varied somewhat between the sheep and human experiments (VCM was diluted 1:3 and 1:5 for sheep and human cells, respectively),

Table 2. Efficiency of Neo^R Transfer Into Adult and Fetal Human Hematopoietic Progenitors In Vitro

	Total No. Colonies Enumerated*							
	No G418				2 mg/mL G418			
Cells Cultured	CFU-Mix	CFU-GM	BFU-E	CFU-E	CFU-Mix	CFU-GM	BFU-E	CFU-E
Adult Marrow								
Control	81	644	1,169	2,206†	0 (0)	0 (0)	0 (0)	0 (0)
Transduced	69	555	870	1,968†	0 (0)	9 (1.6)	9 (1.0)	16 (0.8)
Fetal Liver								
Control	192	739	1,347	26,233	0 (0)	0 (0)	0 (0)	0 (0)
Transduced	174	785	1,386	19,996	9 (5.2)	40 (5.1)	65 (4.7)	1,275 (6.4)

Three (adult) or four (fetal) separate experiments, each involving a separate fetal and adult donor, were conducted. Each cell preparation was cultured at 2 to 4 $\times$ 10^5 cells/mL (adult) or 4 $\times$ 10^5 cells/mL (fetal) as described. Transduction was carried out in the absence of IL-3 or GM-CSF.

*Each value represents the total number of colonies assessed in all experiments. Values in parentheses indicate percent resistant colonies.

†Results from two experiments.

Table 3. Effect of Recombinant Human IL-3 and GM-CSF on the Efficiency of Neo^R Transfer Into Adult and Fetal Human Hematopoietic Progenitors In Vitro

	Total No. Colonies Enumerated*							
	No G418				2 mg/mL G418			
Cells Cultured	CFU-Mix	CFU-GM	BFU-E	CFU-E	CFU-Mix	CFU-GM	BFU-E	CFU-E
Adult Marrow								
Control	55	1,447	248	3,488†	0 (0)	0 (0)	0 (0)	0 (0)
Transduced	63	1,209	501	1,844†	0 (0)	72 (6.0)	21 (4.2)	24 (1.3)
Fetal Liver								
Control	186	881	1,358	18,491‡	0 (0)	0 (0)	0 (0)	0 (0)
Transduced	164	1,076	1,212	30,764	15 (9.1)	105 (9.7)	88 (7.3)	1,900 (6.2)

Three (adult) or four (fetal) separate experiments, each involving a separate fetal and adult donor, were conducted. Each cell preparation was cultured at 2 to 4 $\times$ 10^5 cells/mL (adult) or 4 $\times$ 10^5 cells/mL (fetal) as described. All incubations (control and transduction) were carried out in the presence of IL-3 and GM-CSF.

*Each value represents the total number of colonies assessed in the four separate experiments. Values in parentheses indicate percent resistant colonies.

†Results from two experiments.

‡Results from three experiments.

when adult human bone marrow cells were incubated with undiluted VCM, the percentages of G418-resistant progenitors (CFU-Mix, 0%; CFU-GM, 1.6%; BFU-E, 2%; CFU-E, 1%) were not significantly different from those obtained with the diluted preparation of the N2 vector (CFU-Mix, 0%; CFU-GM, 1.6%; BFU-E, 1%; CFU-E, 0.8%). Although exposure of these progenitors to hematopoietic growth factors GM-CSF and IL-3 improved the efficiency of Neo^R transfer/expression (Table 3), sheep progenitors continued to express the gene at significantly higher levels.

Successful postnatal in vivo gene transfer into hematopoietic cells with the expression of the exogenous gene has been achieved in the mouse[2-4,7] but not in primates[9] or dogs.[14] Both the relative ease with which sheep hematopoietic progenitors appear to become transduced with the N2 vector and the higher efficiency of Neo^R transfer/expression into fetal sheep progenitors correlates with the relatively efficient transfer/expression of Neo^R gene in sheep using the in utero gene transfer protocol.[16] These findings combined with other fetal characteristics such as the availability of bone marrow spaces (obviating the need for cytoablative therapy), the absence of immediate demand upon the newly engrafted stem cells (hematopoiesis in the fetus is generally maintained by hepatic/splenic activity), and the generally favorable growth environment of the fetus, were likely contributing factors to the success of the in utero gene transfer protocol.[16] In our hands, the more primitive progenitor CFU-Mix from adult human bone marrow did not express Neo^R activity in vitro. However, adult sheep marrow CFU-Mix exhibited relatively high levels of G418 resistance. A significant percentage of human fetal liver CFU-Mix also survived the addition of G418 to culture. It is possible that the increased numbers of target CFU-Mix (and of other progenitors) assayed in fetal preparations contributed to the greater efficiency of Neo^R transfer. However, increasing the concentration of hematopoietic progenitors, including CFU-Mix, in preparations of adult human bone marrow by accessory cell depletions to levels exceeding those present in fetal liver cells failed to influence the transfer/expression of Neo^R into adult human CFU-Mix (Table 4). Whether or not differences in the proliferative state of the fetal and adult CFU-Mix play a role in this process is not known. The transfer of the exogenous gene to the more primitive hematopoietic stem cells is important for the successful long term expression of the gene in vivo.[1,21] The long term expression of the Neo^R gene observed in sheep after in utero gene transfer suggests that

Table 4. Efficiency of Neo^R Transfer Into Adult Human Hematopoietic Progenitors In Vitro

	Total No. Colonies Enumerated*							
	No G418				2 mg/mL G418			
Cells Cultured	CFU-Mix	CFU-GM	BFU-E	CFU-E	CFU-Mix	CFU-GM	BFU-E	CFU-E
Whole marrow								
Control	92	2,074	760	—	0	0 (0)	0 (0)	0 (0)
Transduced	123	934	1,148	3,414	0	13 (1.4)	10 (0.9)	9 (0.3)
MO and T depleted marrow								
Control	299	4,605	8,711	16,888	0	0 (0)	0 (0)	0 (0)
Transduced	384	3,519	6,783	10,611	0	12 (0.3)	14 (0.2)	41 (0.4)

Three separate experiments, each involving a separate donor, were conducted. Each cell preparation was cultured before (2 to 4 $\times$ 10^5 cells/mL) and after (0.5 to 4 $\times$ 10^5 cells/mL) depletion of adherent monocyte/macrophages (MO) and T lymphocytes. Transduction was carried out in the absence of IL-3 and GM-CSF.

*Each value represents the total number of colonies assessed in the three separate experiments. Values in parentheses indicate percent resistance colonies.

the transduction of at least some multipotent stem cells had occurred.[16]

Except for diseases such as adenosine deaminase (ADA) deficiency, where a natural pressure for the growth of the treated cells may exist,[1] most postnatal gene therapy attempts will likely require cytoablation of the patient's marrow, with all the risks that this procedure involves. In addition, there are a number of inherited metabolic diseases (eg, Lesch-Nyhan, Tay Sachs) that can produce irreversible damage to the fetus prior to birth. The greater efficiency of gene transfer into fetal hematopoietic progenitors, and the feasibility of the in utero gene transfer protocol in most large animal species, suggest that gene therapy in utero may be a reasonable therapeutic option in some of these disorders.

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