Retroviral mediated gene transfer into bone marrow progenitor cells: use of beta-galactosidase as a selectable marker

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ABSTRACT

Recombinant retroviruses have been utilized as vectors for gene transfer in model systems of gene therapy. Since many of these model systems require the transplantation of genetically modified primary cells it is important to devise methods which will allow the rapid and efficient selection for transplantation of only the cells which are capable of expressing high levels of the transferred gene. This report describes the use of beta-galactosidase as such a selectable marker. Bone marrow progenitors are infected with a recombinant retrovirus encoding beta-galactosidase. Using a fluorescence assay for beta-galactosidase we demonstrate that it is possible to use cell sorting to enrich for cells which will form bone marrow colonies that express high levels of beta-galactosidase. This rapid and non-toxic selection of bone marrow cells may facilitate attempts to achieve gene therapy in a variety of model systems.

INTRODUCTION

An intensively studied model system for gene therapy utilizes the hematopoietic reconstitution of irradiated mice with genetically modified syngenic bone marrow (1-5). For this approach to be successful very efficient gene transfer into bone marrow stem cells must occur. This is particularly important since a majority of the mature blood cells in a reconstituted mouse are derived from one or a few bone marrow stem cells (6,7). The derivation of most blood cells from a small number of stem cells also makes it important that each stem cell be capable of giving rise to progeny expressing high levels of the transferred gene.

Because of their ability to transduce genes at high efficiency, recombinant retroviruses have been widely utilized in the transfer of genes into primary cells, including bone marrow cells. Nevertheless, less than 100% efficiency of infection is often obtained, and in the absence of selection it may be difficult to obtain populations of cells which are expressing the transduced gene. Moreover, even in the presence of selection there is no

rapid way to select for cells that are expressing high levels of the transferred gene.

Currently available selectable markers are often difficult to utilize in primary cells. Antibiotic resistance often requires prolonged growth in culture and the primary cells may either not survive long enough for selection or may change biological properties during the selection process (e.g. differentiate). In a previous report we have described the use of cell surface antigens as selectable markers in primary cells (8). In that study flow cytometry and cell sorting were utilized for rapid selection of cells infected with the recombinant retrovirus. In addition, cells selected for high levels of expression of the selectable marker were shown to express high levels of an adjacent unselected gene. Therefore, this type of rapid quantitative selection allows the generation of populations of cells expressing high levels of both the selectable marker and an adjacent gene product. For purposes such as the investigation of gene transfer into different primary cell types or the study of different retroviral vectors, such cell surface selectable markers have many advantages. However, for use as selectable markers on cells which are to be transplanted into a new host there are several potential disadvantages of this approach. These potential disadvantages include the immunogenicity of the marker as well as the possibility that the marker gene product will alter the homing or function of the transplanted cell.

To circumvent some of these potential problems we have explored the use of intracellular proteins as selectable markers in primary cells. This report describes the use of E.coli beta-galactosidase as a selectable marker in primary hematopoietic cells. Using a newly described fluorescence assay for beta-galactosidase (9) we demonstrate gene transfer and subsequent selection based upon quantitative beta-galactosidase expression in primary human and murine hematopoietic cells.

MATERIALS AND METHODS

Production of Recombinant Retroviruses

The recombinant retrovirus used in this study is a derivative of the HSG-neo vector described by Guild et al. (10). HSG-neo

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DNA was digested with Bam HI and the E.coli beta-galactosidase gene was inserted into the retroviral DNA backbone. The resultant plasmid termed HSG- β -gal, is schematically shown in Figure 1. Plasmid DNA was mixed with PSV₂neo and transfected into the Ψ cri and Ψ cre amphotropic and ecotropic packaging cell lines as described previously (8,11). G418 resistant cell lines were selected and assayed for retrovirus production as described previously (8,11). Retrovirus titer was determined by X-gal staining of NIH 3T3 cells infected with the recombinant retrovirus. No replication competent retrovirus was detected in the supernatants of these packaging cell lines.

Retroviral Infection

Human bone marrow cells were obtained from healthy donors with approval of the institutional review board. Murine bone marrow was obtained from the femurs of Balb/C mice. Infection of the bone marrow cells was done by co-culture with mixed amphotropic and ecotropic packaging cell lines producing the HSG- β -gal virus. Mixed amphotropic and ecotropic packaging cell lines were utilized because prior results with murine bone marrow cells revealed improved rates of infection with this protocol (Strair, R.K., unpublished observation). Three days prior to infection the amphotropic and ecotropic producer cell lines were mixed 3:1 and allowed to grow to confluence for 48 hours. The mixed packaging cell lines were then split 1:10 and the following day 5×10^6 bone marrow cells were added for co-culture in the presence of 2 μ g/ml polybrene. Forty eight hours later the non-adherent bone marrow cells were harvested and assayed for beta-galactosidase activity as described below.

Beta-galactosidase Assay

Bone marrow cells were assayed for beta-galactosidase using a fluorescence assay with fluorescein di-beta galactopyranoside (FDG) (Molecular Probes, Eugene, Oregon) as a substrate. Conditions for staining the cells were those described by others (9) and as per the manufacturers recommendations. Cells were assayed by flow cytometry on a FACScan (Becton-Dickinson, Mountain View, California). For experiments requiring cell sorting cells were stained with FDG and cell sorting was performed on a FACStar cell sorter (Becton-Dickinson). Histologic staining for beta-galactosidase was performed by staining with 5-bromo 4-chloro 3-indolyl beta-galactoside (X-gal) as described by Price et al. (12).

Bone Marrow Culture

Bone marrow cells were mixed with bone marrow culture media containing PHA-LCM, erythropoietin, and methylcellulose. The bone marrow media was obtained from the Terry Fox Laboratory, Vancouver, British Columbia. Bone marrow cultures were grown for ten days at 37° and 5% CO₂ prior to staining.

For X-gal staining an equal volume of a 2 $\times$ concentrated X-gal staining solution was added to the plates and the plates were incubated at 37° for 12 hours. Colonies that contained greater

than 32 cells were scored, colonies were scored as positive if greater than 20% of the cells in the colony were blue after 12 hours of X-gal staining.

RESULTS

The recombinant retroviral vector used in these studies is a derivative of the histone HSG-neo vector described by Guild et al. It contains an internal histone H4 promoter driving the E. coli beta-galactosidase gene and a deletion in the 3'LTR. Amphotropic and ecotropic packaging cell lines producing high titers ($>10^5$ /ml of cell culture media) of this virus were generated and used in co-culture experiments with either primary human or primary murine bone marrow cells. Mixed amphotropic and ecotropic packaging cell lines were used for co-culture with bone marrow cells because prior results using murine bone marrow indicated higher rates of infection using the mixed amphotropic and ecotropic culture (Strair, R.K. unpublished observation). Although the mechanism by which this increased rate of infection of murine bone marrow occurs has not been established, the mixed amphotropic and ecotropic culture was used for infection of both murine and human bone marrow in these studies. We have not yet determined if the mixed amphotropic and ecotropic culture results in better infection of human bone marrow cells when compared to the rates of infection seen with amphotropic packaging lines alone. The bone marrow cells were co-cultured with the packaging cell lines for 48 hours, harvested and analyzed for beta-galactosidase activity twenty four hours later. Figure 2 demonstrates that the co-culture conditions utilized resulted in the efficient transfer of the beta-galactosidase gene into primary hematopoietic cells. Using a fluorescence assay for beta-galactosidase approximately 80% of the bone marrow cells can be shown to express beta-galactosidase. Staining of this same population of cells with 5-bromo 4-chloro 3-indolyl beta-galactoside (X-gal) demonstrated infection of approximately 20% of the cells (data not shown). This greater sensitivity of the

Figure 1. Plasmid DNA was digested and modified as described in Materials and Methods. The resultant HSG- β -gal plasmid is schematically shown. The notch in the 3' LTR indicates a deletion of the enhancer element.

Figure 2. Human bone marrow cells were obtained from a healthy donor and either mock infected or infected with the HSG- β -gal virus as described in Materials and Methods. At 72 hours the non-adherent bone marrow cells were stained with FDG and analyzed for beta-galactosidase activity by flow cytometry. Cell number is displayed on the ordinate and relative fluorescence is displayed on the abscissa. Light line, mock infected cells; dark line, infected cells.

fluorescence assay as opposed to the histochemical assay has been described previously (9). Thus, it appears that although the majority of the cells have been infected and express low levels of beta-galactosidase, only 20% of the cells are expressing enough beta-galactosidase to be detected by X-gal staining. This is similar to the infection of primary human and murine lymphocytes infected under similar conditions (13).

Immediately after co-culture bone marrow cells were incubated for 10 days in semi-solid bone marrow culture and stained with X-gal. Figure 3a shows the microscopic appearance of a colony which does not stain with X-gal and figure 3b shows the microscopic appearance of a colony which does stain with X-gal. These results demonstrate that it is possible to detect colonies expressing high levels of beta-galactosidase by examination of plates containing colonies stained with X-gal. Using these techniques approximately 3–5% of bone marrow colonies are stained with X-gal after infection of bone marrow with the retrovirus containing the beta-galactosidase gene sequences. This is in contrast to the 20% of the cells which stain with X-gal (and 80% of the cells which stain with FDG) prior to bone marrow culture. Thus it appears that although efficient gene transfer into bone marrow cells has occurred, the majority of colonies do not express high enough levels of beta-galactosidase to stain with X-gal. This may reflect relatively inefficient infection of colony forming cells, instability of expression of beta-galactosidase, or the modulation of expression during cell differentiation in culture.

To determine if beta-galactosidase expression can be used as

Figure 3. Human bone marrow cells were infected with the HSG- β -gal virus and propagated in bone marrow culture for 10 days. The plate of cells was then stained with X-gal and individual colonies were photographed in situ.

a selectable marker in primary bone marrow progenitors, bone marrow was co-cultured with packaging cell lines producing the recombinant retrovirus. Three days after infection cells were stained with FDG and populations with high and low levels of fluorescence were generated by cell sorting. Figure 4 shows the relative fluorescence of unsorted and sorted bone marrow populations after staining with FDG. To determine if the populations which have been sorted for high level expression of FDG have a greater number of cells which will form colonies

Figure 4. Murine or human bone marrow cells were infected with the HSG- β -gal virus, stained with FDG and subjected to cell sorting to generate populations of bone marrow cells with high and low levels of beta-galactosidase expression. Two independent experiments are demonstrated, in each case FDG staining of the starting population (pre-sort) is depicted in the top panel and the FDG staining of the 2 sorted populations prior to bone marrow culture is depicted in the bottom panels. 'Low' refers to cells sorted for low levels of beta-galactosidase activity, 'High' refers to cells sorted for high levels of expression. Cell number is displayed on the ordinate and relative fluorescence is displayed on the abscissa. The right panel depicts an experiment performed with human bone marrow, the other experiment was performed with murine marrow.

Figure 5. Murine bone marrow was infected with the HSG- β -gal virus was stained with FDG and sorted into populations of cells with low levels of fluorescence and high levels of fluorescence. These populations of cells were then grown in bone marrow culture for 10 days and stained with X-gal. Left panel, bone marrow culture of cells sorted for low levels of fluorescence after FDG staining. Right panel, bone marrow culture of cells sorted for high levels of fluorescence after FDG staining.

Table I. Bone marrow cells were infected with the HSG- β -gal virus, stained with FDG and sorted into populations with high and low fluorescence. These populations were cultured in semi-solid bone marrow culture media and the culture plates were stained with X-gal. Experiments I and III were with murine bone marrow. Experiment II was with human bone marrow. Total colony number on each plate was between 37 and 127, plating efficiency for the sorted populations of cells were equivalent, as described in the text.

	% of Colonies Staining with X-gal		
	Experiment I	Experiment II	Experiment III
Unsorted	3.2	2.5	3.8
Low Fluorescence	0	0	0
High Fluorescence	22	18	25

with high levels of beta-galactosidase, each population was grown in semi-solid bone marrow culture for 10 days and stained with X-gal. Figure 5 shows the macroscopic appearance of these bone marrow culture plates stained with X-gal. Macroscopic colonies expressing high levels of beta-galactosidase are easily detectable in the population sorted for high FDG fluorescence. Cell sorting results in populations of cells that are generally 40%–60% viable, as determined by trypan blue exclusion data.

The three experiments shown in Table I demonstrate that these viable populations of cells obtained after cell sorting for FDG expression are capable of growing in culture and giving rise to colonies, some of which express enough beta-galactosidase to stain with X-gal. Furthermore the population of cells which has been sorted for high level FDG expression has a 6 fold enrichment for X-gal stained colonies. Colony formation is equally efficient in each of the sorted populations with colonies developing at a frequency of 0.025%–0.050% of the viable bone marrow cells plated. These results indicate that cell sorting of FDG stained cells results in a population of viable cells which have been enriched for cells that will form colonies that have high enough levels of beta-galactosidase to be detected by X-gal staining.

DISCUSSION

A standard approach to increasing the expression of genes transferred by recombinant retroviruses has been to modify the regulatory elements of the retrovirus vector. While this has resulted in significant advances and it is now possible to demonstrate reconstituted mice with expression of the transferred gene, it would be very beneficial to have a means of rapidly selecting for transplantation only those cells which will give rise to progeny expressing high levels of the transferred gene.

In an attempt to develop such a selection scheme this report has described the use of beta-galactosidase as a selectable marker in primary bone marrow cells. The results indicate that after infection with a retrovirus encoding beta-galactosidase bone marrow cells can be stained with a fluorescent substrate for beta-galactosidase, that the stained cells can be sorted into populations of cells which express high and low levels of beta-galactosidase and that the cells sorted for higher levels of beta-galactosidase are enriched for cells that will form colonies that express enough beta-galactosidase to stain with X-gal. The cells sorted for lower levels of FDG fluorescence are depleted of cells that give rise to colonies that stain with X-gal. Since the fluorescence assay is much more sensitive than X-gal staining it is possible that many of the colonies that do not stain with X-gal are expressing low

levels of beta-galactosidase. This is suggested by the data shown in figure 2 which indicates that 80% of the bone marrow cells express beta-galactosidase when assayed by FDG fluorescence but only 20% stain with X-gal. Alternatively the cells initially sorted for increased FDG fluorescence may have down regulated beta-galactosidase expression during their differentiation in culture or only transiently expressed high levels of beta-galactosidase. Clarification of the status of those colonies which are derived from cells sorted for high FDG fluorescence but do not express enough beta-galactosidase to stain with X-gal requires a method for either applying the fluorescence stain to colonies in situ or for the detection of beta-galactosidase DNA in colonies using PCR. Regardless of the results of those experiments it appears that fluorescence staining and cell sorting can enrich for cells that give rise to colonies with high levels of expression.

Since prior experiments have demonstrated that cells selected for high levels of expression of a selectable marker also express high levels of an adjacent unselected gene, the experiments presented here may form a basis for the use of selectable markers in primary cells such as bone marrow. Additional studies will need to be done to determine if cells selected for very high levels of expression of the marker maintain that high level of expression for a long period of time. In addition it will be necessary to study the modulation of expression with cell differentiation. Using currently available techniques it should be feasible to undertake these studies.

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