Flowcytometry for Estimating Efficient Transfection of Bone Marrow Cells with BCR-ABL Gene

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ABSTRACT:

BACKGROUND:

Chronic myeloid leukaemia can be accurately modeled in laboratory mice by the retroviral transfer of a BCR-ABL gene into murine hematopoietic stem and progenitor cells, followed by transplantation of these cells into irradiated recipient mice.

OBJECTIVE:

The use of Retroviral vector for transfer of BCR-ABL gene into murine bone marrow cells (BMC) and measurement of efficiency of transfection by flowcytometry.

METHODS:

Murine bone marrow cells obtained from mice were cultured in a medium containing the supernatant of BCR-ABL (p210) transfected platinum E cells which is rich in retroviral vector carrying the BCR-ABL (p210) gene.The vector express green fluorescent protein (GFP) as well so that the efficiency of transfection of murine BMC with the target gene was able to be measured using flowcytometry.

RESULTS:

The use of the retrovirus packaging cell line enhanced the transduction of BMC with the retroviral vector and efficiency of transfection was 72% as measured by the flowcytometry.

CONCLUSION:

Transfer of BCR-ABL gene into murine BMC by retroviral vector that carry GFP marker which allowed the estimation of transfection efficiency by the flowcytomery.

KEY WORDS: BCR-ABL gene, bone marrow cells, GFP.

INTRODUCTION:

Chronic myeloid leukemia (CML) is a stem cell disease, in which neoplastic cells carry a translocated Philadelphia chromosome, in which a hybrid BCR-ABL gene encodes a fusion oncoprotein, BCR-ABL, with constitutive tyrosine kinase activity. BCR-ABL induces multiple signaling pathways to transduce the oncogenic signal, which ultimately results in uncontrolled proliferation and neoplastic expansion [(1)]. Three major BCR-ABL fusion proteins have been described in patients (p185, p210, and p230), and these are associated with three different clinical phenotypes of BCR-ABL disease (acute lymphoblastic leukemia, CML, and CML characterized by slower progression kinetics, respectively)[(2)]. In vivo experiments in mice, showed that all three BCR-ABL1 translocation products (p185, p210, and p230) were able to transform 5-Fluorouracil (5-FU) enriched bone marrow cells and cause a similar phenotype (CML-like disease) in recipient mice [(2,3,4,)].

Murine models of CML have not only greatly enhanced our understanding of leukemogenesis but also of physiologic human hematopoiesis and have been indispensable for preclinical drug testing of BCR-ABL inhibitors [(1,3)]. Several technical approaches were used to generate mouse models of CML-like disease: injection of cell lines or primary cells from CML patients into recipient mice, transduction of bone marrow- derived cells with retroviral vectors that carry the BCR-ABL cDNA with subsequent transplantation into lethally irradiated recipient mice or generation of transgenic mice expressing the oncogene [(2,5)].

The aim of this study is to transfer the BCR-ABL gene to murine BMC using a retroviral vector that carry a fluorescent marker in order to analyze the results by the flowcytometry.

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MATERIALS & METHODS:

The basic protocol details the transfection of Platinum E cells (a retroviral packaging cell line) with plasmid carrying the BCR-ABL (P210) gene, then the retroviral particles in the supernatant were used to transducer murine BMC (bone marrow cells) that can be used for creating a CML murine model(3,6). All the materials were supplied by the American University of Beirut, where the whole work was done in 2011.

Transfection of Platinum E cells with the plasmid:

Platinum E cells were seeded at a rate of 700 000 cells per well of 6-well plates in MEM (minimum essential medium) (Lonza), eight hours later, Platinum E cells were transfected with the 3ug plasmid (MIGRp210 plasmid DNA) expressing the BCR-ABL gene and coding for GFP (green fluorescent protein), for transfection Calcium phosphate method was used (6) and a control wells were not treated with gene transfer. Wells were incubated at 37C overnight. Next day medium was removed from the wells and replaced by fresh medium, the plates incubated at 37C overnight. Retroviral supernatant from transfected Platinum E cells was collected and filtered so it can be used to transduce the BMC.

Bone marrow cell (BMC) harvest:

mice (age 6-10 weeks) were injected IV with 5-FU (3mg/20g weight). These mice were left for 4 days before sacrificing them and extracting their bone marrow (6). mice were killed and the long bones were cut (2 tibias and 2 femurs per mouse). The harvested bones were dipped in MEM on ice during the sacrifice procedure. the bone marrow cavity of the long bones were flushed by aspirating media through the bones using a 27gauge-needle for the tibias and a 22gauge-needle for the femurs, until the bone turns white. All the steps were done on ice. The collected flushed medium were passed through strainer to trap large particles and debris and allow the flow-through of the bone marrow cells into a new sterile collecting tube. The tube then Centrifuged (1500 rpm for 5 minutes) and 10ml RBC lysis buffer were added per pellet of BMC and Incubated on ice to allow appropriate lysis of the RBC and centrifuged to collect the whitish pellet. The BMC pellet was first prestimulated in culture for 24 h. by Resuspending it in 15-20ml of MEM containing (15%FBS, 1% Penicillin/Streptomycin, 0.2% kanamycin , 1% Sodium pyruvate, IL-3, IL-6 and stem cell factor (SCF))cells then were counted by trypan blue staining.

Transduction of the BMC:

Infecting the BMC with the filtered fluid that carry the retroviral vector (which now carry the p210 gene) by one single round of spinoculation. To every 10^7 BMC, 2ml of 10% FBS-MEM, 2ml viral supernatant (retroviral vector) and 8ug/ml polybrene were added and the mixture were loaded onto a 6-well plate, Centrifuged for 90 minutes at 2750rpm and 32˚C to increase transduction efficiency. When centrifugation is done, the plate(s) incubated at 37˚C for around 2 hours before resuspending the cells in a tube, centrifuging the tube and collecting the pellet. The pellet was resuspended in cold sterile HBSS (Hanks buffered salt solution) (Lonza) at a concentration of 1 million cells per 150-200ul.

Flowcytometry:

(FACScan; Becton Dickinson) : after resuspening the pellet, a sample was taken to be read by the flocytometry. Transfected and non transfected murine BM cells were analyzed for forward scatter (FSC) and side scatter (SSC), and expression of GFP determined at FL1 channel.

Statistical analysis:

Fluorescence was analyzed on with Cell Quest software (Becton Dickinson, San Jose, CA). and the results were expressed as percentages.

RESULTS:

Multipotent hematopoietic progenitor cells are rare in the bone marrow population, so using a retoviral vector for BCR/ABL gene transfer, the retroviruses must be of sufficient titer to infect these rare cells. To consistently generate high-titer BCR-ABL (p210) expressing retroviruses, we used Platinum E retroviral packaging cells. In this work, the evidences of transfection of these clls had determined by two observations, the first one is the plaque formation. Plaques are formed when the retrovirus infects a cell, then the infected cell will lyse and spread the infection to adjacent cells where the infection-to-lysis cycle is repeated, the infected cell area will create a plaque (an area of infection surrounded by uninfected cells). Living cells (non infected) were stained with Eosin, leaving the infected

cells transparent, which can be seen visually or with an optical microscope (figure 1A). The second evidence of transfection of Platinum E cells was the GFP expression as a green flouresence within the cytoplasm of transfected cells using flourescent microscope (figure 1B). Green fluorescent protein (GFP) here used as as gene marker so that we can measure how many murine BM cells transfected with BCR-ABL gene. As shown in figure two, for flowcytometric analysis of GFP, ten thousands events (cells) were gated electronically according to the control (nontransfected cells) for forward-scatter (FSC) and side-scatter (SSC) properties to include the main population of the cells and exclude dead cells. Then and because the flouresence emitted by GFP adsorb at 488nm, we had to choose FL1 channel to set the control in gate one (M1). All the transfected murine bone marrow cells were then shifted to the right and being localized in gate two (M2). The percentage of cells in gate two (M2) were analyzed by the software to be given as percentage . More than half of the number of the murine BM cells (72%) were gated in M2 indicating an efficient transfection.

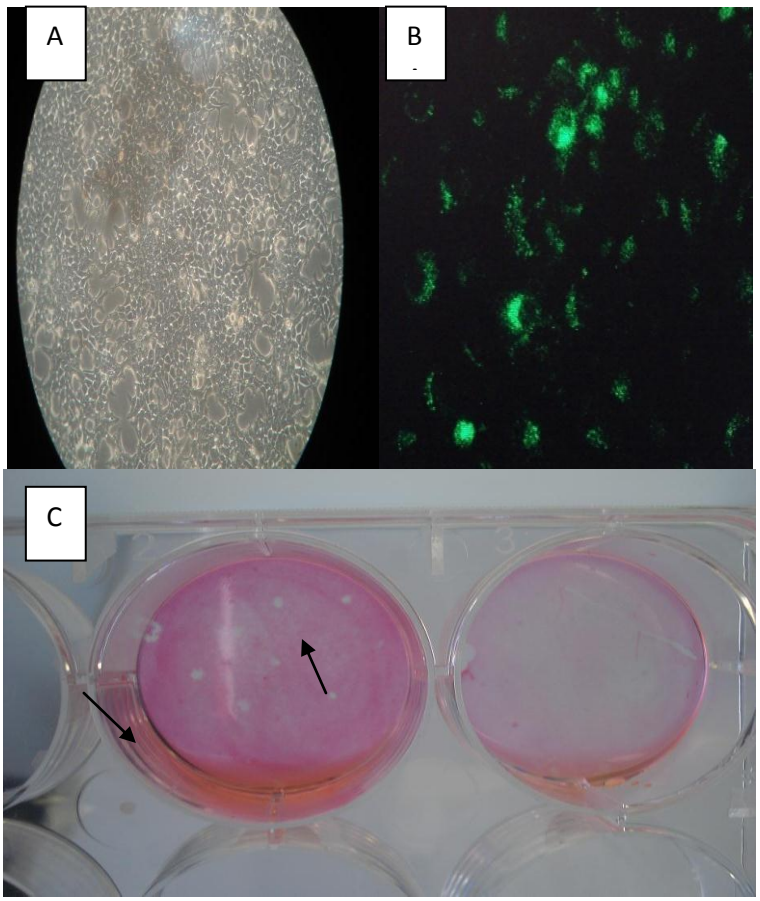
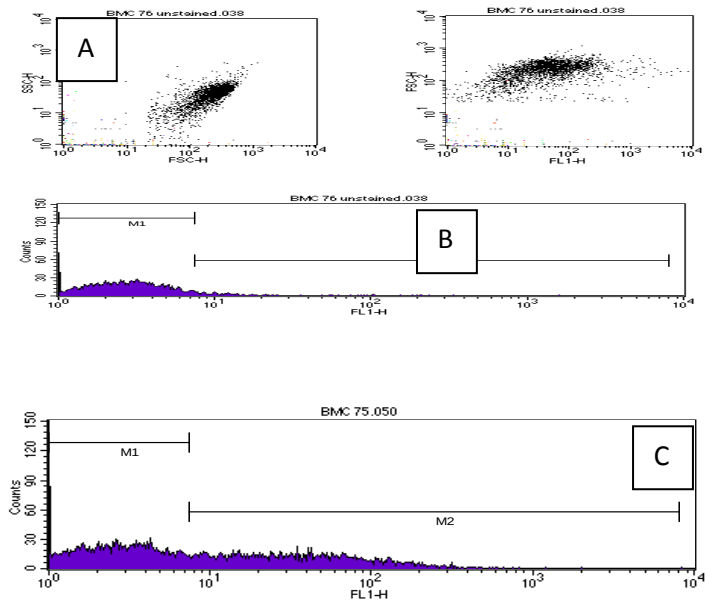


Figure 1: (a)Light microscopy showing the monolayer of Platinum E cells ; (b) Fluorescent microscopy showing the green fluorescence with in the cytoplasm of the cells reflecting the presence of the plasmid inside the cells; (c) showing the plaques (the cells that are infected with the retovirus).



Figur 2: Flowcytometry for the murine bone marrow cells (BMC):

a- Dot plot to localize the population of the BMC and exclude any debris
b- Control BMC (non transfected cells), all are localized in M1 gate
c- BCR-ABL transfected BMC, the shift to the right indicate the presence of GFP reflecting the transfection with BCR-ABL gene and all the cells within the M2 gate are transfected.

DISCUSSION:

Although much has been learned about the biology of BCR/ABL through many studies, a complete understanding of the pathophysiology of BCR/ABL associated leukemias requires the expression of the oncogene in the hematopoietic system of a living organism. This is because the complex nature of leukemia cannot be adequately modeled in any currently existing cell culture system [2,4]. Human CML can be faithfully modeled in mice by retroviral transduction of the BCR/ ABL gene into mouse bone marrow cells, followed by transplantation into irradiated syngeneic mice [2,5].

In this work and for efficient transfection of murine BM cells, a transient transfection of the retroviral packaging cell line was applied. retroviral vectors and packaging cells are important tools for gene transfer applications, the introduction of retroviral vectors containing the gene of interest into suitable packaging cells enables the production of infectious retroviruses, and these particles can infect murine BM cells stably and transmit the BCR-ABL gene into chromosomes [7]. In our work The Platinum-E (Plat-E) Cell Line was chosen rather than other conventional packaging cell lines, because it is a potent retrovirus packaging cell line with a high yield retroviral structure protein expression (gag, pol, env). Plat-E cells produce retroviruses with an average titer of 1 x 10^7 infectious units/mL by transient transfection. In addition, only coding sequences of viral structural genes are used, avoiding any unnecessary retroviral sequences [6,7]. Pretreatment of mice with 5 FU (5-Fluorouracil) was recommended to kill dividing cells and recruits haemopoeitic stem cells into the cell cycle [6] in order to collect the haemopoeitic stem cells and subject them for gene transfer, however, a more advanced technique now applied for identifying and isolating hematopoietic stem cell (HSC) by the use of a set of defined surface markers in combination with high-speed cell sorting techniques which allowed for the efficient sorting of rare cell populations [2].

The green fluorescent protein (GFP) has been introduced as a promising reporter for monitoring gene transfer and expression in BM cells. It is a protein with an intrinsic fluorescence permitting its detection without the addition of fluorogenic compounds so replacing the need for labeling the cells with any fluorescent marker. This protein used here as gene marker giving an indication for efficiency of transfection of murine bone marrow cells with our target gene (BCR-ABL gene). The use of GFP has preclude any requirement for secondary molecules, such as antibodies or substrates, for analysis of expression by flowcytometry or even fluorescence microscopy and therefore limits time and effort necessary for the detection of the transduced cells.In addition, the fluorescence intensity differences between GFP-positive and GFP-negative murine BM cells are relatively high, thus facilitating and improving the efficiency of the flowcytometric selection of retrovirally transduced cells [3,9,10]. The feasibility of using GFP as a marker for the selection of retrovirally transduced target cells was evaluated for BM cells in many studies and found to be advantageous above the use of other selectable markers such as, eg, the bacterial β-galactosidase gene, as the relatively high endogenous β-galactosidase activity in some cell types and the requirement for transporting fluorogenic substrates across the cell membrane while maintaining cell viability, have limited its application [8,9,11].

CONCLUSION:

It seemed that the transfer of BCR-ABL gene was successful using retroviral vector that carry GFP marker which allowed rapid detection of BCR-ABL transduced murine BM cells by flowcytomery.

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