The AFT024 stromal cell line improves MDR1 gene transfer to CD34+ cells derived from human umbilical cord blood

HUALING ZHANG, ZEQING WEN*, SHOUMIN LAN, CHANGZHONG LI, JIANFENG LI, XIAOJING ZHANG

Shandong Provincial Hospital Shandong University, Jinan 250021, PR. China, Department of Obstetrics & Gynecology, Shandong Provincial Hospital, 324 Jingwu Road, Jinan, Shandong Provincial Hospital Shandong University, Jinan 250021, PR. China. Email: wenzeqing@hotmail.com

Received May 5, 2006

Human hematopoietic stem cells (HSCs) are difficult to transfect with retroviral vectors because of their quiescent nature. Based on the theory that the murine fetal stromal cell line AFT024 can recruit significant numbers of HSC into cell cycle without loss of their primitive function, we transduced human umbilical cord blood cells (UCB) derived $CD34^+$ cells with a retroviral vector pHaMDR1/A containing the human multidrug resistant 1 gene (MDR1) during co-culture with the AFT024 feeders. We found that the presence of the AFT024 cells increased the proportion of Rh-123^{dull} cells up to 35.5%±11.4% and transduced colony-forming cells (CFCs) up to 15.2%. Six weeks after transplantation of 5×10^4 day 0 uncultured $CD34^+$ HSCs or their equivalents expanded in the presence or absence of the AFT024 cells for 21 days into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, we found that $CD34^+$ cells expanded in the presence of the AFT024 cells engrafted in each receptor mouse and the percentage of $CD45^+$ cells reached 18.8%±9.5%, of which 18.1%±6.0% were Rh-123^{dull} cells. These results suggest that the AFT024 stromal cells can significantly improve MDR1 gene transfer efficiency and maintain the engrafting ability of the $CD34^+$ HSCs derived from UCB.

Key words: AFT024 cell line; Gene therapy; Multidrug resistance; Umbilical cord blood cells transplantation

The human multidrug resistance 1 (MDR1) gene is a candidate gene encoding the membrane-located drug-efflux pump P-glycoprotein. P-glycoprotein confers resistance to a wide array of cytostatic agents, such as paclitaxel and etoposide by pumping these drugs out of the cells. [1-3] The transfers of MDR1 genes into hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) are expected to confer drug-resistance to the bone marrow (BM) of patients so as to protect them from myelotoxicity which is the main adverse effect of high-dose chemotherapy.

In the initial murine studies and human clinical studies, transplantation of MDR1 gene-modified primitive hematopoietic cells have demonstrated chemoprotection *in vivo*. [4-7] However, low gene transfer efficiency *ex vivo* and a low number of reconstituting MDR1 gene-made cells and/or inefficient expression of the transgene *in vivo* has hampered its wide application in clinical trials. The difficulty is that host cell division is a prerequisite for the murine retrovirus to integrate into its genome while human hematopoietic cells are mostly quiescent in nature. [8, 9] Published optimized protocols demonstrated the improvement of the level of gene transfer into human HSCs. These optimizations include: the use of alternate vectors, such as pseudotyped vectors (GALV envelope[10] or feline endogenous retrovirus envelope[11,12]) and lentivirus vectors (HIV vectors[13,14]); the use of early acting cytokines, such as stem cell factor(SCF)[15] , Flt-3 ligand (FL)[16] ,and thrombopoietin (TPO)[17]; and the use of recombinant fibronectin colocalizating retroviral particles and target cells[18,19]. Prestimulation of HSCs with cytokines almost becomes a routine procedure in HSCs transduction with retroviral vectors. However, these stimulatory signals forcing HSCs into the cell cycles result in impaired long-term engraftment *in vivo*. [20] So how to induce HSCs into cell cycles without impairing their engraftment ability will be an appealing attempt.

Inadequate cell source for HSCs transplantation is another problem for the wide application of gene transfer in clinical

*Correspondence to: Department of Obstetrics & Gynecology, Shandong Provincial Hospital, 324 Jingwu Road, Jinan, Shandong 250021, People's Republic of China. E-mail: wenzeqing@hotmail.com

trials. The umbilical cord blood (UCB) becomes an attractive alternative to bone marrow as a target for gene-therapy applications. It contains a high number of primitive progenitor cells with greater *ex vivo* expansion ability and *in vivo* engraftment capability compared with bone marrow and mobilized peripheral progenitor cells. [21, 22] Moreover, the lower alloreactivity of human UCB contributes to a lower incidence of graft-vs-host disease (GVHD) after transplantation. [23, 24] However, the finite number of hematopoietic stem and progenitor cells is a limiting factor for the use of UCB as a graft for adult transplant recipients. An ideal strategy to resolve this problem is to increase the cell number available from a single UCB unit by *ex vivo* expansion while maintaining their engraftment capability. This strategy has been proved to be successful in HSCs by applying various cytokines combinations and/or stromal feeder cell layers. [22, 25, 26] The AFT024 feeder cells have been shown to better maintain and even expand human UCB $CD34^+$ repopulating cells than other stromal cell lines. [27, 28]

The murine fetal liver cell line AFT024 has been shown to maintain long-term repopulating murine stem cells for up to 7 weeks ex vivo. [29] It can also expand primitive hematopoietic progenitors from UCB for 5 weeks. [30] The mechanism of the AFT024 feeder cells to expand the HSCs has been characterized. They can recruit significant numbers of HSCs into cell cycles and increase asymmetric divisions of HSCs which result in a balance between self-renewal and differentiation. [31] Thus, it would be possible to introduce retroviral markers into HSCs efficiently when co-cultured with the AFT024 cells. In this paper, we isolated $CD34^+$ cells from UCB and co-cultured them in the presence and absence of the AFT024 feeder cells for 7 days. Then in the subsequent 14 days, the MDR1 retrovirus was added to infect cultured $CD34^+$ cells in the presence or absence of the AFT024 feeder cells. 5×10^4 day 0 uncultured $CD34^+$ cells or their equivalents expanded for 21 days were transplanted into NOD/SCID mice. We investigate whether the presence of the AFT024 cells can improve the MDR1 gene transfer efficiency and the engraftment of expanded cells in NOD/SCID mice.

Material and methods

Generation of MDR1 retroviral packaging cells and production of retroviral supernatant

Recombinant retroviral plasmid pHaMDR1/A (kindly provided by Dr. Michael Gottesman, Laboratory of Cell Biology, NCI, NIH, USA) containing the full-length human MDR1 cDNA was transfected into PA317 packaging cell line (ATCC) with Lipofectamine2000 (Invitrogen) according to manufacturer's recommendations. Clones of transfected PA317 packaging cells were selected in colchicine (60 ng/mL) expanded at 32°C and retrovirus-containing supernatant was harvested and filtered through a 0.45μm filter. The supernatant was further centrifuged at 14,000g for 2 hours at 4°C. The precipitated retroviruses were resuspended in Iscove's modified Dulbecco's Medium (IMDM; Gibco-BRL) and stored at -80°C until use. The retrovirus titer was determined by infection of NIH3T3 cells. [32]

Isolation of $CD34^+$ cells from human UCB

UCB samples were obtained from the Department of Obstetrics, Shandong Provincial Hospital (Jinan, China). After obtaining maternal donor informed consent, fresh blood was collected from the umbilical cord vein by standard procedures used for UCB banking. Mononuclear cells (MNCs) were separated by Ficoll-paque plus (Amersham) density gradient centrifugation. $CD34^+$ cells were isolated using the MACS CD34 Progenitor Cell Isolation Kit (Miltenyi) according to the manufacturer's instruction. The purity of $CD34^+$ cells was determined by flow cytometry (Beckman Coulter) using CD34-FITC monoclonal antibody (Miltenyi) to assess the percentage. Isotype controls were used in every experiment.

Culture of the AFT024 stromal cells

The AFT024 cells (kindly provided by Dr. Kateri A. Moore, Princeton University, Princeton, NJ, USA) [29] were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) and 50 μM β-mercaptoethanol (2-ME) at 33°C, 5% CO_2, and 100% humidity. Cells were grown in 24-well plates coated with 1% gelatin (Sigma) and confluent monolayers were irradiated at 20Gy using linear accelerator (Siemens) 24 hours before used as a feeder layer. [33]

Virus infection of $CD34^+$ cells

Immediately after isolation, 1×10^5/mL fresh $CD34^+$ cells were seeded into 24-well transwell inserts (0.4 μm pore size, Costar) above the AFT024 feeders in the bottom compartment. This primary culture medium consisted of IMDM, 10% FBS, 10% horse serum, 50 ng/mL recombinant human stem cell factor (rhSCF; R&D), 50 ng/mL flt3-ligand (FL; R&D), 50 ng/mL recombinant human interleukin-3 (rhIL-3; Biosource), 20 ng/mL human thrombopoietin (TPO; Biosource), 10 ng/mL recombinant human interleukin-7 (rhIL-7; R&D), 100 U/mL penicillin(Sigma), and 100 μg/mL streptomycin (Sigma). On day 7 and day 14 of incubation, cells were harvested from the wells, counted and analyzed for progenitor cell content ($CD34^+$ cells and colony-forming cells). Secondary culture was initiated with a concentration of 5×10^5/ml in new transwell inserts coated with 20μg/cm^2 of the recombinant fibronectin fragment CH-296 (RetroNectin; Takara Shuzo) which was bound with pre-loaded retrovirus according to the manufacturer's recommendation. Cells were fed twice per week with fresh medium and retrovirus supernatant. After 21 days of culture, cells was harvested and resuspended in fresh medium without retrovirus for 24 hours before further analysis.

Rh-123 efflux assay

MDR1 gene expression was determined in MDR1-transduced $CD34^+$ cells and in NOD/SCID repopulating cells (SRCs) by their ability to efflux the fluorescent dye rhodamine-123 (Rh-123; Sigma), resulting in a Rh-123^{dull} phenotype. [34]

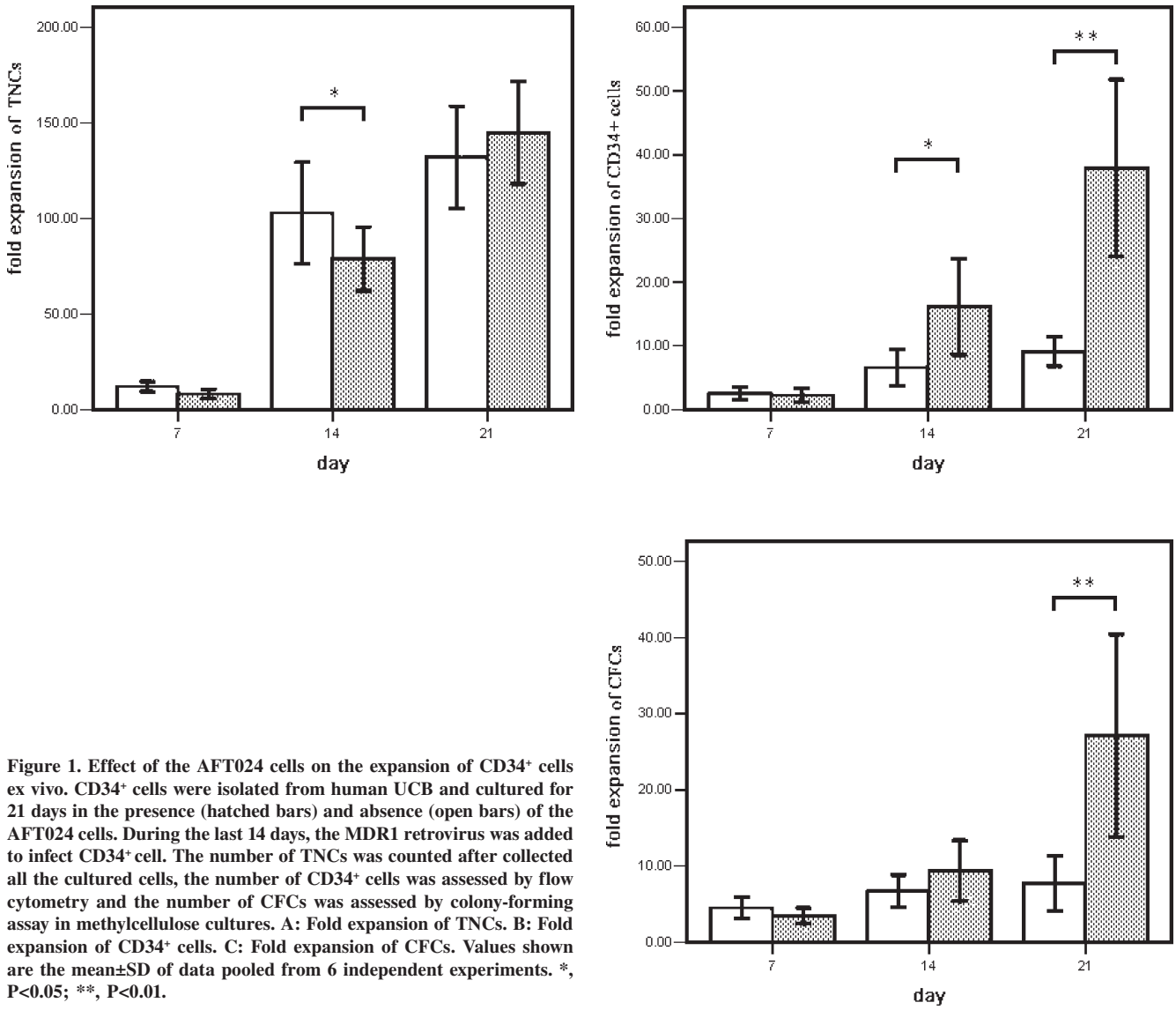

Figure 1. Effect of the AFT024 cells on the expansion of CD34⁺ cells ex vivo. CD34⁺ cells were isolated from human UCB and cultured for 21 days in the presence (hatched bars) and absence (open bars) of the AFT024 cells. During the last 14 days, the MDR1 retrovirus was added to infect CD34⁺ cell. The number of TNCs was counted after collected all the cultured cells, the number of CD34⁺ cells was assessed by flow cytometry and the number of CFCs was assessed by colony-forming assay in methylcellulose cultures. A: Fold expansion of TNCs. B: Fold expansion of CD34⁺ cells. C: Fold expansion of CFCs. Values shown are the mean±SD of data pooled from 6 independent experiments. *, P<0.05; **, P<0.01.

One million cells were washed and resuspended in IMDM containing 5% FCS and Rh-123 at a final concentration of 0.2 μg/mL. After an incubation period of 30 minutes at 37°C, cells were centrifuged, resuspended in IMDM containing 5% FCS, and further incubated for 60 minutes at 37°C to allow the cells to efflux the loaded Rh-123. Immediately before flow cytometric measurement, propidium iodide staining (Sigma; final concentration, 1 μg/mL) was done to exclude dead cells from the analysis.

Colony-forming cells (CFCs) assay

Freshly isolated and transfected CD34⁺ cells were tested for the presence of CFCs in methylcellulose cultures in the presence or absence of paclitaxel (10 ng/mL). Triplicated cell samples were plated in 24-well plate at 1×10^3 cells per well in 1mL in IMDM medium containing 1.3% (wt/vol) methylcellulose (Sigma), 15% FBS, 15% horse serum, 4 mM L-glutamine, 50mM 2-ME, 100 ng/mL rhSCF, 50 ng/mL IL-3, 50 ng/mL IL-6, 20ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Biosource), and 4 U/mL human erythropoietin (EPO; Biosource). After incubated at 37°C with 5% CO_2, 100% humidity for 12 to 14 days, colony-forming units (CFUs) were enumerated.

Animals

NOD/SCID mice were kept in isolators under pathogen-free conditions in Animal Laboratory of Shandong University, Jinan, China. 24 hours before transplantation, 36 6- to 8-week-old female mice were conditioned by sublethal irradiation with a total dose of 3 Gy. These mice further received 5×10^4 day 0 uncultured human CD34⁺ HSCs isolated from human UCB or their equivalents expanded in the presence or absence of

the AFT024 cells for 21 days, described in the previous paragraphs. These cells were transplanted into mice in a volume of 300 μL of PBS by lateral tail vein injection.

Analysis of engraftment

Mice were sacrificed by cervical dislocation 8 to 12 weeks after transplantation of human cells. BM cells were flushed with IMDM from femurs and tibias of each mouse and were treated with hemolytic buffer (150 mmol/L ammonium chloride, 12 mmol/L sodium bicarbonate, and 0.1 mmol/L EDTA) for lysis of erythrocytes. Single-cell suspensions from BM were labeled with anti-human CD45-PC5 antibody (BD Bioscience) for 30 minutes at 4°C and analyzed by flow cytometry.

Statistics

Results of experimental points from different experiments were reported as mean±standard deviation (SD). Significance levels were determined by paired two-sided Student's t-test analysis as indicated.

Results

The AFT024 Cells improve CD34⁺ cells ex vivo expansion

To investigate the $CD34^+$ cells *ex vivo* expansion, we isolated human UCB-derived $CD34^+$ cells by a MACS kit and analyzed their qualities. A median of 4×10^8 mononuclear cells (MNCs) was obtained from each UCB unit and a median of 4×10^6 of $CD34^+$ cells was obtained from these MNCs by MACS kit isolation. The purity of $CD34^+$ cells averaged 80.7% determined by flow cytometry using a FITC-conjugated anti-CD34 monoclonal antibody. Approximately 0.8% mononuclear cells isolated from UCB were $CD34^+$ cells. The percentage of $CD34^+$ cells from UCB correlated with 1% reported by Mayani H and Lansdorpb PM. [35]

First we tested the effect of the AFT024 cells on the expansion of total nucleated cells (TNCs). Figure 1A shows the fold increase of TNCs when $CD34^+$ cells were cultured either with or without the AFT024 feeders. From day 0 to day 14, the proliferation rate of TNCs in the absence of the AFT024 cells was higher than that in the presence of the AFT024 cells and on day 14 TNCs expanded (102.9±26.6) fold when cultured alone, while only (78.9±16.6)fold when co-cultured with the AFT024 cells. However, after day 14, TNCs co-cultured with the AFT024 feeders began to proliferate more effectively than those without the AFT024 feeders. By day 21, TNCs in the AFT024 co-culture group expanded (145.0±26.8) fold, higher than (132.0±26.7) fold in the absence of the AFT024 cells, while there was no difference between two groups (P>0.05). This result indicates that the AFT024 cells don't affect the TNCs expansion.

Next we want to examine the $CD34^+$ cells expansion cultured in the presence or absence of the AFT024 cells. As can be seen in Figure 1B, on day 7, the fold expansion of $CD34^+$ cells was similar between the two groups. Then from that time point, $CD34^+$ cells co-cultured with the AFT024 feeders began to proliferate rapidly, especially after day 14. On day 21, compared with day 0, $CD34^+$ cells co-cultured with the AFT024 cells expanded (37.9±13.9) fold while $CD34^+$ cells cultured alone expanded only(9.1±2.3) fold.($P<0.01$)

We also assessed the expansion of CFCs in the presence and absence of the AFT024 cells. As shown in Figure 1C, on day 7, CFCs expanded (4.5±1.4) fold in the absence of the AFT024 cells and (3.5±1.0) fold in the presence of the AFT024 cells($P>0.05$). To day 14, CFCs expanded more in the presence of the AFT024 cells (9.4±4.0 fold) than in the absence of the AFT024 cells(6.7±2.1 fold) . These results indicated that the presence of the AFT024 improved the expansion of CFCs from day 7 on. Yet there was no significant difference in the CFCs expansion fold on day 14 between two groups ($P>0.05$). Until day 21, a significant difference ($P<0.01$) between two groups was found in the fold number of CFCs expansion. Compared with day 0, the total number of CFCs was expanded up to (27.1±13.3) fold in the presence of the AFT024 feeders, while only (7.7±3.6) fold in the absence of the AFT024 feeders.

Taken as a whole, we found that the AFT024 stromal cells strongly improve $CD34^+$ cells and CFCs expansion but not TNCs expansion *ex vivo*.

The AFT024 cells enhance the gene transfer efficiency ex vivo

After $CD34^+$ cells were cocultured with the AFT024 feeders for 7 days, cells were transferred to CH-296-coated transwells and transduced with MDR1 retrovirus supernatant for 14 days. The expression of P-glycoprotein in MDR1-transduced $CD34^+$ cells was determined by measuring the efflux of the fluorescent dye Rh-123. The proportion of $Rh\text{-}123^{dull}$ cells was (35.5±11.4)% when cells were transduced in the presence of the AFT024 feeder and (16.6±3.2)% in the absence of the AFT024. There was significant difference between the two groups ($P<0.01$)

To assess the efficiency of MDR1 gene transfer into CFCs, we plated the tranduced cells into methylcellulose-based semisolid medium supplemented with 10 ng/mL of paclitaxel. The gene transfer efficiency was evaluated by measuring the percentage of surviving drug-resistant colonies (the total CFCs presented in the absence of paclitaxel set as 100%). 46% of CFC transduced in the presence of the AFT024 cells and only 15.2% in the absence of the AFT024 cells displayed drug resistance ($P<0.01$). Taken as whole, these data indcate that the level of gene transfer in $CD34^+$ cells and CFCs can be enhanced by the presence of the AFT024 feeders.

Effect of the AFT024 cells on the engraftment of human cells in NOD/SCID mice

To evaluate the repopulating ability of human UCB derived HSCs or their expanded equivalent, six weeks after transplanting the samples into the NOD/SCID mice, the mice were sacrificed and BM cells were obtained and analyzed for the percentage of human $CD45^+$ leukocytes. Mice with less than 0.1% human $CD45^+$ cells detected were considered to have nonengraftment. 5×10^4 day 0 $CD34^+$ cells engrafted 9 of 12 mice and the percentage of $CD45^+$ cells was 4.1%±2.3%. All the mice receiving day 21 expanded cells in the presence of the AFT024 cells showed detectable $CD45^+$ cells with a percentage of 18.8%±9.5%. By contrast, $CD45^+$ cells were

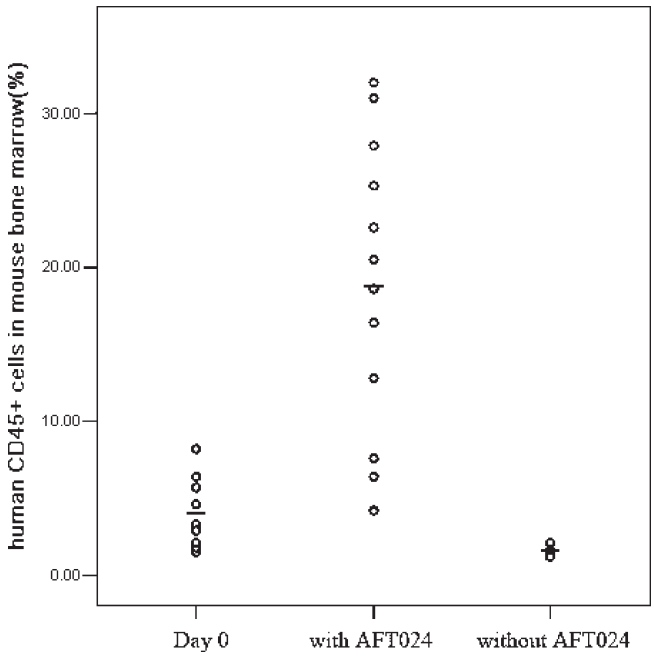

Figure 2. Effect of the AFT024 cells on the engraftment of human cells in NOD/SCID mice. We transplanted 5×10⁴ day 0 uncultured CD34⁺ HSCs or their equivalents expanded in the presence or absence of the AFT024 cells for 21 days into NOD/SCID mice. After six weeks, we detected the percentage of CD45⁺ cells in the bone marrow of mice. Dots represent individual animals. Horizontal bars indicate the average per group.

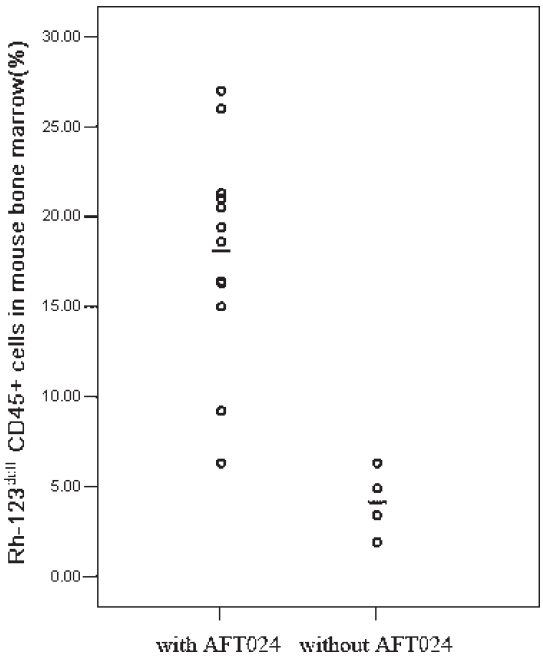

Figure 3. Effect of the AFT024 on the transduction of SRCs. Six weeks after transplantation of transduced cell in the presence or absence of the AFT024 cells into NOD/SCID mice, we detected the percentage of Rh-123^{dull} CD45⁺ cells in the bone marrow of mice. Dots represent individual animals. Horizontal bars indicate the average per group.

detected in only 4 of 12 mice receiving the 21-day expansion equivalent of 5×10⁴ CD34⁺ cells in the absence of the AFT024 cells and the highest percentage was 2.1%. (Fig. 2). These results suggest that human HSCs expanded in the presence of AFT024 cells can better engraft into NOD/SCID mice than day 0 uncultured HSCs and expanded cells in the absence of AFT024 cells.

Effect of the AFT024 cells on the transduction of SRCs

The transduced SRCs were evaluated by the proportion of CD45⁺ cells with Rh-123 efflux function in the BM of mice that have received MDR1-transduced CD34⁺ cells. The percentage of Rh-123^{dull} CD45⁺cells was determined by flow cytometry. As shown in Figure 3, the percentage of Rh-123^{dull} CD45⁺ cells in the group receiving transduced cells in the presence of the AFT024 was 18.1%±6.0%, significantly higher than a proportion of 4.1%±1.9% in the 4 mice receiving transduced cells in the absence of the AFT024 ($P<0.01$). Schiedlmeier et al [36] found a tight correlation between MDR1 gene marking determined by Quantative PCR and the proportion of Rh-123^{dull} human CD45⁺ leukocytes, thus we predicate from the higher percentage of Rh-123^{dull} CD45⁺ cells that the AFT024 cells enhanced MDR1 gene transfer efficiency in SRCs.

Discussion

It is much easier to transduce murine HSCs than human HSCs using retrovirus vectors in standard short-term transduction protocols. [37] The far more quiescent nature of human HSCs than murine HSCs is one important reason to explain this. Murine HSCs divide at lease once even during a few days of transduction period while human HSCs may not divide at all for several weeks. A very attractive idea to use the Dexter-type long-term bone marrow cultures (LTBMC) for gene transfer was put forward in respect that the proliferation of primitive cells during these cultures for many weeks would provide enough chances for retrovirus vectors to integrate into the cells. However, investigators have demonstrated that this protocol is ineffective for both transduction efficiency and engraftment. [38] The AFT024 feeders have been demonstrated to allow the HSCs in G_0 phase to be recruited to cycles without loss of primitive function by increased asymmetric divisions. [31] Here we studied the feasibility of a 3-week transduction protocol with the support of the AFT024 feeders.

We achieved the MDR1 gene transfer efficiency of up to 46% in CFCs in the presence of the AFT024 cells, much higher than 15.2% in the absence of the AFT024 cells. van der Loo JC et al also reported that prestimulation of HSCs in the pres-

ence of the AFT024 for 4 days increased the number of CFCs by 3.5 fold compared to cultures without AFT024 cells. [39] However, using the 3-week LTBMC transduction, only 22.2% of CFCs were transduced with the retroviral vector containing GFP gene. [38] In their study, cell recovery on day 21 was only 21.3% and CFCs recovery was only 9.7% as compared with the input cells on day 0. Thus the low level of gene transfer efficiency in LTBMC might be caused by the loss of primitive cells in the process of weekly removal of the non-adherent cells and by the differentiation of primitive cells during the long-term culture. In our studies, we harvested both adherent and non-adherent cells for secondary cultures. On day 21, $CD34^+$ cells and CFCs were expanded for 37.9 fold and 27.1 fold respectively in the presence of the AFT024 cells. The augment of the absolute number of $CD34^+$ cells and CFCs caused by the AFT024 feeders may contribute to the increased MDR1 gene transfer efficiency. The proliferation rate of total cells are probably not in close relation with gene transfer efficiency, as the expansion fold of TNCs showed no significant differences between with and without the AFT024 cells. Thus, the expansion of primitive hematopoietic cells by the AFT024 cells may play a more important role in the increased gene transfer efficiency.

The effective gene transfer into HSCs is attractive to apply this optimized protocol, while the more important criterium is, that whether these cells responsible for long-term *in vivo* engraftment, can be effectively transduced. Encouragingly, we achieved an average of 18.1% in MDR1 transfer rate of *in vivo* repopulating cells detected in all the 12 mice transplanted with the day 21 cells cultured in the presence of the AFT024 cells. Transplantation of the day 21 expansion equivalent of 5×10^4 $CD34^+$ cells in the presence of the AFT024 cells succeeded in engraftment in all mice with a higher percentage of $CD45^+$ cells detected compared with either day 0 cells transplantation or day 21 culture without the AFT024 cells. This finding was in line with the effect of the AFT024 cells on the maintenance and even expansion of transplantable primitive hematopoietic cells reported by other investigators. [29, 30, 40] The higher cell dose transplanted in the AFT024 group also contributes to the engraftment of human cells since the long-term engraftment of human cells was cell dose-dependent. [41]

The murine retrovirus carrying the target gene can integrate into cells only in the period of cell divisions, whereas the ideal target cells of gene therapy, that's the most primitive human HSC, are quiescent or have very slow cycling rates under natural conditions. [42, 43] So, in order to facilitate retroviral gene insertion, HSCs has to be recruited into cell cycles. Cytokines supplemented in cultures could stimulate HSCs into cell cycle and facilitate the retroviral transduction, while disappointingly, they lead to an engraftment defect in the host. [20] Stromal cells and adhesion to the recombinant fibronectin fragment CH-296 could counteract the loss of long-term engraftment potential of HSCs induced by cytokines. [44, 45, 46] In our study, both groups involved the use of CH-296-coated culture plate while a much higher engraftment of human cells was observed in the presence of the AFT024 cells group compared to the absence of the AFT024 cells. This result suggests that the AFT024 feeders played a more important role than CH-296 in rescuing the engraftment ability of those repopulating cells induced into cell cycle transit. The AFT024 cell line has been shown to have superior ability to support *ex vivo* survival and maintenance of long-term engraftable human hematopoietic progenitors than human stromal cells and other murine stromal cell line. [27, 28] The AFT024 feeders can recruit significant number of primitive cells into cell cycles and increase the absolute number of cells undergoing asymmetric divisions. [31] These effects can not be induced by cytokines combination alone. [47, 48] Even the myeloid-lymphoid-initiating cells (ML-IC) which were almost exclusively quiescent could be induced to asymmetric divisions by the AFT024 cells. [31] Most studies provide evidence for the association between asymmetric divisions and primitive function *ex vivo*. [47.48.49] So we speculate that there would be more chances for MDR1-retrovirus to integrate into the primitive stem cells when asymmetric divisions occur. More effective MDR1 gene transduced primitive cells would result in higher level of transduced SRCs.

There are some hypotheses about the mechanism responsible for the supportive effect of the AFT024 cells on HSCs. The AFT024 cells were believed to provide a microenvironmental niche through direct contact with HSCs to maintain a balance between their self-renewal and differentiation. [29] Other investigators proved that the AFT024 cells can also maintain the primitive functions of HSCs even in non-contact culture system. [40] This indicates that the supportive effect of the AFT024 cells can be carried out by secreting soluble factors. Moore KA et al have identified more than one hundred kind of factors secreted only by the AFT024 cells, but not by other stromal cells that does not have the ability to maintain primitive cells. Though it is still unknown that how each of these factors acts on HSCs, their supportive effect on HSCs is sure. It has been demonstrated that the supernatant collected from the AFT024 cells culture or the medium supplemented with some of these factors, such as large 6-O-sulfated heparan sulfate proteoglycans, can replace the AFT024 cells for supporting HSCs. [39]

A major focus in the clinical UCB transplantation is to expand HSCs to an enough number for an adult use while maintaining all of the characters of HSCs at the same time. The maintenance and expansion of HSCs depends on the culture conditions used. In consideration of the expansion of $CD34^+$ cells and CFCs which can represent partly the primitive HSCs and HPCs, the AFT024 cells greatly increased the fold-expansion of them. This findings is consistent with the documented effect of the AFT024 cells on the maintenance of primitive hematopoietic cells. [29, 30, 40] Our studies used a population of UCB cells enriched for the surface marker CD34 because the majority of current HSCs transplants were performed using $CD34^+$-enriched cells. Assay of the expansion of $CD34^+$ cells could direct clinical utility. The average number of $CD34^+$ cells obtained from one UCB sample is

4×10^6. After expansion of 3 weeks, we achieved 1.5×10^8 of $CD34^+$ cells (37.9 fold) which is sufficient to transplant a 75kg patient (2×10^6 $CD34^+$ cells/kg body weight). Stimulated only by cytokines, though the total number of expanded cells was slightly lower than those co-cultured with the AFT024 feeders, most HSCs were driven to the road of differentiation in long-term cultures so that low level of $CD34^+$ cells expanded could not satisfy the clinical use.

In summary, our studies proved that the AFT024 cell line can significantly improve the gene transfer efficiency *ex vivo* and the transduced cells maintain the engraftment ability *in vivo*. Furthermore, the 21-day culture protocol expands the $CD34^+$ cells derived from UCB to an enough level for adult transplantation. Although it is still in a long way, this protocol proves a potential method to transfect MDR1 gene into HSCs derived from human UCB with retroviral vector.

This work was supported by NO. 023130104, Key Fundamental Research Program, Department of Science and Technology of Shandong Province.

References

[1] CHAUDHARY PM, RONINSON IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell. 1991;66:85-94

[2] GOTTESMAN MM, PASTAN I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem. 1993;62:385-427

[3] BOESEN JJB, NOOTER K, VALERIO D. Circumvention of chemotherapy-induced myelosuppression by transfer of the mdr1 gene. Biotherapy. 1994;6:291-302.

[4] O'SHAUGHNESSY JA, COWAN KH, NIENHUIS AW, et al. Retroviral mediated transfer of the human multidrug resistance gene (MDR-1) into hematopoietic stem cells during autologous transplantation after intensive chemotherapy for metastatic breast cancer. Hum Gene Ther. 1994 Jul;5(7):891-911.

[5] HANANIA EG, FU S, RONINSON I, et al. Resistance to taxol chemotherapy produced in mouse marrow cells by safety-modified retroviruses containing a human MDR-1transcription unit. Gene Ther. 1995;2:279-284

[6] DEVEREUX S, CORNEY C, MACDONALD C, et al. Feasibility of multidrug resistance (MDR-1) gene transfer in patients undergoing high-dose therapy and peripheral blood stem cell transplantation for lymphoma. Gene Ther. 1998;5(3):403-408.

[7] HESDORFFER C, AYELLO J, WARD M, et al. Phase I trial of retroviral-mediated transfer of the human MDR1 gene as marrow chemoprotection in patients undergoing high-dose chemotherapy and autologous stem-cell transplantation.J Clin Oncol. 1998 Jan;16(1):165-172.

[8] MILLER DG, ADAM MA, MILLER AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol. 1990;10:4239-4242

[9] LEWIS PF, EMERMAN M. Passage through mitosis is required for oncoretroviruses but not for human immunodeficiency virus. J Virol. 1994;68:510-516

[10] KIEM HP, HEYWARD S, WINKLER A, et al. Gene transfer into marrow repopulating cells: comparison between amphotropic and gibbon ape leukemia virus pseudotyped retroviral vectors in a competitive repopulation assay in baboons. Blood. 1997 ;90(11):4638-4645.

[11] GATLIN J, MELKUS MW, PADGETT A, et al. Engraftment of NOD/SCID mice with human CD34(+) cells transduced by concentrated oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. J Virol. 2001;75(20):9995-9999.

[12] KELLY PF, DONAHUE RE, VANDERGRIFF JA, et al. Prolonged multilineage clonal hematopoiesis in a rhesus recipient of CD34 positive cells marked with a RD114 pseudotyped oncoretroviral vector. Blood Cells Mol Dis. 2003;30(1):132-143.

[13] MIYOSHI H, SMITH KA, MOSIER DE, et al. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. Science. 1999;283(5402):682-686.

[14] GUENECHEA G, GAN OI, INAMITSU T, et al. Transduction of human $CD34^+$ $CD38^-$ bone marrow and cord blood-derived SCID-repopulating cells with third-generation lentiviral vectors. Mol Ther. 2000;1(6):566-573.

[15] DUNBAR CE, SEIDEL NE, DOREN S, et al. Improved retroviral gene transfer into murine and Rhesus peripheral blood or bone marrow repopulating cells primed in vivo with stem cell factor and granulocyte colony-stimulating factor. Proc Natl Acad Sci U S A. 1996;93(21):11871-11876.

[16] TISDALE JF, HANAZONO Y, SELLERS SE et al. Ex vivo expansion of genetically marked rhesus peripheral blood progenitor cells results in diminished long-term repopulating ability. Blood 1998;92:1131-1141.

[17] KIEM HP, ANDREWS RG, MORRIS J et al. Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor. Blood. 1998 Sep 15;92(6):1878-1886.

[18] MORITZ T, PATEL VP, WILLIAMS DA. Bone marrow extracellular matrix molecules improve gene transfer into human hematopoietic cells via retroviral vectors. J Clin Invest. 1994;93(4):1451-1457.

[19] MORITZ T, DUTT P, XIAO X et al. Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. Blood. 1996;88(3):855-862.

[20] KITTLER EL, PETERS SO, CRITTENDEN RB, et al. Cytokine-facilitated transduction leads to low-level engraftment in nonablated hosts. Blood. 1997; 90(2):865-872.

[21] HAO Q-L, SHAH AJ, THIEMANN FT et al. A functional comparison of $CD34^+CD38^-$ cells in cord blood and bone marrow. Blood.1995;86: 3745-3753.

[22] BARKER JN, WAGNER JE. Umbilical-cord blood transplantation for the treatment of cancer. Nat Rev Cancer. 2003;3 :526–532.

[23] WAGNER JE, BARKER JN, DEFOR TE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of $CD34^+$ cell dose and HLA disparity on treatment-related mortality and survival. Blood. 2002; 100:1611 –1618 .

[24] BARKER JN, WEISDORF DJ, DEFOR TE, et al. Rapid and complete donor chimerism in adult recipients of unrelated donor umbilical cord blood transplantation after reduced-intensity conditioning. Blood.2003; 102:1915–1919.

[25] SHIH CC, HU MC, HU J, MEDEIROS J, FORMAN SJ. Long-term ex vivo maintenance and expansion of transplantable human hematopoietic stem cells. Blood. 1999; 94:1623–1636.

[26] ANDO K, NAKAMURA Y, CHARGUI J, et al. Extensive generation of human cord blood $CD34^+$ stem cells from Lin^- $CD34^-$ cells in a long-term in vitro system. Exp Hematol. 2000; 28:690–699.

[27] NOLTA JA, THIEMANN FT, ARAKAWA-HOYT J, et al. The AFT024 stromal cell line supports long-term ex vivo maintenance of engrafting multipotent human hematopoietic progenitors. Leukemia. 2002;16(3):352-361.

[28] PUNZEL M, MOORE KA, LEMISCHKA IR, et al. The type of stromal feeder used in limiting dilution assays influences frequency and maintenance assessment of human long-term culture initiating cells. Leukemia. 1999; 13(1):92-7.

[29] MOORE KA, EMA H, LEMISCHKA IR. In vitro maintenance of highly purified, transplantable hematopoietic stem cells. Blood. 1997; 89:4337–4347.

[30] LEWIS ID, VERFAILLIE CM. Multi-lineage expansion potential of primitive hematopoietic progenitors: superiority of umbilical cord blood compared to mobilized peripheral blood. Exp Hematol. 2000; 28(9):1087-1095.

[31] PUNZEL M, LIU DH, ZHANG T, et al. The symmetry of initial divisions of human hematopoietic progenitors is altered only by the cellular microenvironment. Exp Hematol. 2003; 31:339–347.

[32] KOTANI H, NEWTON PB 3RD, ZHANG S, et al. Improved methods of retroviral vector transduction and production for gene therapy. Hum Gene Ther, 1994,5:19-28.

[33] PUNZEL M, WISSINK SD, et al. The myeloid-lymphoid initiating cell (ML-IC) assay assesses the fate of multipotent human progenitors in vitro. Blood. 1999; 93(11): 3750-3756.

[34] FRUEHAUF S, BREEMS DA, KNAAN-SHANZER S, et al. Frequency analysis of multidrug resistance-1 gene transfer into human primitive hematopoietic progenitor cells using the cobblestone area-forming cell assay and detection of vector-mediated P-glycoprotein expression by rhodamine-123. Hum Gene Ther. 1996;7(10): 1219-1231.

[35] MAYANI H, LANSDORP PM. Biology of human umbilical cord blood-derived hematopoietic stem/progenitor cells. Stem cell. 1998; 16(3): 153-165

[36] SCHIEDLMEIER B, SCHILZ A, KUHLCKE K, et al. Multidrug resistance 1 gene transfer can confer chemoprotection to human peripheral blood progenitor cells engrafted in immunodeficient mice. Hum Gene Ther. 2002; 13: 233-242

[37] HANAZONO Y, TERAO K, OZAWA K. Gene transfer into nonhuman primate hematopoietic stem cells: implications for gene therapy. Stem Cells. 2001;19(1): 12-23.

[38] RELANDER T, FAHLMAN C, KARLSSON S, et al. Low level of gene transfer to and engraftment of murine bone marrow cells from long-term bone marrow cultures. Exp Hematol. 2000; 28:373–381.

[39] VAN DER LOO JC, LIU BL, GOLDMAN AI, et al. Optimization of gene transfer into primitive human hematopoietic cells of granulocyte-colony stimulating factor-mobilized peripheral blood using low-dose cytokines and comparison of a gibbon ape leukemia virus versus an RD114-pseudotyped retroviral vector. Hum Gene Ther. 2002;13(11):1317-1330.

[40] LEWIS ID, ALMEIDA-PORADA G, DU J, et al. Umbilical cord blood cells capable of engrafting in primary, secondary, and tertiary xenogeneic hosts are preserved after ex vivo culture in a noncontact system. Blood. 2001;97:3441–3449.

[41] SCHIEDLMEIER B, KUHLCKE K, ECKERT H.G, et al. Quantitative assessment of retroviral transfer of the human multidrug resistance 1 gene to human mobilized peripheral blood progenitor cells engrafted in nonobese diabetic/severe combined immunodeficient mice. Blood. 2000; 95:1237–1247.

[42] CHENG T, RODRIGUES N, SHEN H, et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science 2000;287: 1804–1808.

[43] MAHMUD N, DEVINE SM, WELLER KP, et al. The relative quiescence of hematopoietic stem cells in nonhuman primates. Blood 2001;97: 3061–3068.

[44] DAO MA, HASHINO K, KATO I, et al. Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells. Blood. 1998; 92:4612-4621.

[45] DONAHUE RE, SORRENTINO BP, HAWLEY RG, et al. Fibronectin fragment CH-296 inhibits apoptosis and enhances ex vivo gene transfer by murine retrovirus and human lentivirus vectors independent of viral tropism in nonhuman primate CD34+ cells. Mol Ther. 2001; 3(3):359-367.

[46] WU T, KIM HJ, SELLERS SE, et al. Prolonged high-level detection of retrovirally marked hematopoietic cells in nonhuman primates after transduction of CD34+ progenitors using clinically feasible methods. Mol Ther. 2000; 1(3):285-293.

[47] HUANG S, LAW P, FRANCIS K, et al. Symmetry of initial cell divisions among primitive hematopoietic progenitors is independent of ontogenic age and regulatory molecules. Blood. 1999; 94:2595–2604.

[48] PUNZEL M, ZHANG T, LIU D, et al. Functional analysis of initial cell divisions defines the subsequent fate of individual human $CD34^+CD38^-$ cells. Exp Hematol. 2002; 30(5):464-472.

[49] BRYDER D, JACOBSEN SE. Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. Blood. 2000;96:1748–1755.