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Removal of Inhibitory Substances with Recombinant Fibronectin-CH-296 Plates Enhances the Retroviral Transduction Efficiency of $CD34^+CD38^-$ Bone Marrow Cells

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In retroviral gene transduction, the efficiency of viral infection was reduced by the proteoglycans and some other materials secreted by the producer lines. In order to remove these inhibitors we have developed the rFN-CH-296–facilitated protocol. Because the rFN-CH-296 molecule has strong ability to bind a retroviral vector, rFN-CH-296 bound plates are utilized to wash out the unbound putative inhibitors present in a virus supernatant. The gene transduction efficiencies of human $CD34^+CD38^-$ BMCs with a GALV-pseudotyped vector and the rFN-CH-296–facilitated protocol were compared with the protocol without a coating plate with CH-296, the mean gene transduction efficiencies being found to be 95.5 and 71.1%, respectively.

Key words: bone marrow cells, CH-296, gene transfer, gibbon ape leukemia virus, retrovirus vector.

Retroviral vectors are currently one of the most widely used systems for gene transduction, in both experimental studies and clinical trials (*1, 2*). In particular, murine leukemia virus (MLV) has been traditionally used as the vector of choice for clinical gene therapy protocols, and a variety of packaging systems (*3, 4*) and viral production systems (*5, 6*) have been developed.

When murine-based packaging cell lines derived from NIH/3T3 were used for retroviral production, the efficiency of the viral infection and transduction were reduced by the proteoglycans secreted by the murine based packaging cell lines including parental NIH/3T3 cells (*7*). Forestell *et al.* (*8*) also demonstrated that the amphotropic envelope from a packaging line contains some materials that inhibit viral infection. To overcome these problems, a human-derived packaging cell line that produces a high titer viral supernatant has been developed (*9*). Purification of a retroviral vector was also attempted using a low speed centrifugation procedure to remove undesirable substances in the viral supernatant and to concentrate the retrovirus vector (*10, 11*).

We and others have already demonstrated that a recombinant fibronectin fragment was an efficient tool for enhancing gene transfer into hematopoietic stem cells using a retroviral vector system (*12*). The recombinant fibronectin fragment (rFN-CH-296) (*13*), which contains three functional domains, *i.e.* the cell-binding domain (C-domain), heparin-binding domain (H-domain), and CS-1 sequence, enhances retroviral mediated gene transduction by co-locating target cells and virions on the rFN-CH-296 molecules (*12*). The C-domain and CS-1 sequence interact with target cells through integrin receptors VLA-5 and VLA-4, and a virion can be localized upon the H-domain composed of type III repeats III 12, III 13, and III 14.

Therefore, if we use plates coated with rFN-CH-296 (RetroNectin™), whose H-domain possesses the ability to bind retrovirus, transferable inhibitors from the producing cell line can be washed out from the supernatant of a retrovirus vector (*14, 15*). We describe here the protocol of optimized gene transfer into $CD34^+CD38^-$ bone marrow cells (BMCs) utilizing RetroNectin™ plates and gibbon ape leukemia virus (GALV) envelope pseudotyped retrovirus vector (*16*) carrying the red-shift GFP gene.

Retroviral packaging cell line PG13 (ATCC CRL-10686) carrying the red-shift GFP (Takara Shuzo, Otsu) retrovirus vector was maintained in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Gaithersburg, MD) supplemented with 10% fetal bovine serum (BioWhittaker), and 50 U/ml penicillin and 50 μg/ml streptomycin (GibcoBRL, Life Technologies, Gaithersburg, MD). The GALV vector was prepared by filtering (0.45 μm) the supernatant of the culture fluid of this producer line. The viral titer of this supernatant was 4.6×10^5 infectious-units/ml.

$CD34^+CD38^-$ BMCs were purified by negative immunoselection from 4×10^6 human $CD34^+$ BMCs (BioWhittaker) using anti-CD38 monoclonal antibodies and a MACS separation column (Miltenyi Biotec, Bergish Gladbach, Germany). The collected CD38-negative BMCs were suspended in IMDM (GibcoBRL) supplemented with 20% BIT9500 (Stemcell Technologies, British Columbia, Canada), 10^{-4} mol/liter 2-mercaptoethanol (GibcoBRL), 40 μg/ml human low density lipoproteins (Sigma, St Louis, MO), 100 ng/ml human flt-3/flk-2 ligand (FL; R&D Systems, Minneapolis, MN), 100 ng/ml human stem cell factor (SCF; Genzyme, Cambridge, MA), 20 ng/ml human interleukin-3 (IL-3; Genzyme), 100 ng/ml human IL-6 (Pepro Tech EC, London, England), and 100 ng/ml human soluble IL-6 receptor (sIL-

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Abbreviations: rFN, recombinant fibronectin; BMCs, bone marrow cells; GALV, gibbon ape leukemia virus; GFP, green fluorescent protein; SN, supernatant; BV, bound virus.

6R; R&D Systems). $CD34^+CD38^-$ BMCs were cultured at 37°C in 5% CO_2.

Prior to infection, virus bound plates were prepared. Five hundred microliters of the GALV vector was pre-loaded onto a well of a CH-296 (RetroNectin™; Takara Shuzo) coated non-tissue culture-treated 24-well plate. The plate was incubated for 4 h at 32°C. Just prior to infection, the viral supernatant was washed away and 2-day-prestimulated $CD34^+CD38^-$ BMCs were added to each wells ($1–2 \times 10^4$ cells/well) to allow infection. For infection, $CD34^+CD38^-$ BMCs were suspended in 400 μl of the virus supernatant with fresh cytokines [bound virus (BV) + supernatant (SN) infection] or serum-free medium with cytokines (BV infection). As a control, a tissue culture-treated plate was used (*17*) with protamine sulfate (5 μg/ml). Two days after the infection, the cells were harvested and the gene transfer efficiencies were assessed by flow cytometric analysis (FACS Vantage; Becton Dickinson, San Jose, CA) of GFP expression.

The complex of IL-6 and sIL-6R, or Hyper-IL-6 (the fusion protein of IL-6 and sIL-6R) in the presence of SCF significantly stimulates the *ex vivo* expansion of hematopoietic progenitor and stem cells (*17–19*). In addition, this combination strongly induced the GALV envelope receptor pit-1 expression and enhanced the efficiency of gene transfer by the GALV vector (*17*). We described here the effect of the cytokine combination of FL + SCF + IL-3 + IL-6 + sIL-6R on RetroNectin™-assisted gene transfer into human $CD34^+CD38^-$ bone marrow cells, and compared the transfer efficiency of the RetroNectin™ method and that of the method of B. Hennemann *et al.* (*17*).

At first, infection was carried out with bound virus (BV) plus supernatant (SN) on a RetroNectin™ plate or tissue culture plate. There was no significant differences in gene transfer efficiency between the RetroNectin™ transduction method and the method of B. Hennemann *et al.* ($p > 0.1$. Fig. 1A). In the cases of both RetroNectin™ and tissue culture plates, non-diluted SN showed lower transduction efficiency than the 5-times diluted SN. On the other hand, as shown in Figure 1B, 5-times diluted SN infection on RetroNectin™ plate showed much higher gene transfer efficiency. These results indicate that inhibitory materials as to infection may exist in this SN prepared from the culture fluid of a viral producer line. It has been reported that proteoglycans secreted from a murine based packaging line inhibited viral infection (*7*). Our observation also indicated that gene transduction on rFN-CH-296 was inhibited at the infection process by the conditioned media of amphotropic packaging line ψ CRIP or even by its parental NIH/3T3 cells, but the viral localization process on rFN-CH-296 was not inhibited (unpublished data). In the case of PG13, which is also derived from NIH/3T3, this viral supernatant also contained undesirable substances from the culture fluid. Next, $CD34^+$ $CD38^-$ BMCs were infected only with BV, *i.e.* without the addition of SN. When a tissue culture plate was used as a substratum of BV, the gene transfer efficiency was only 40% that of live cells (Fig. 2). In the case of a tissue culture plate, however, BV infection still showed higher efficiency than BV + SN infection.

On the other hand, when infected on RetroNectin™, surprisingly, 80% of live cells and 95% of $CD34^+CD38^-$ cells expressed the red-shift GFP gene. These data indicated that some substrates secreted by the GALV vector producer line inhibit gene transfer and that the inhibitors can be easily washed out from the RetroNectin™ coated dish. We have already demonstrated (*14, 15*) that the RetroNectin™ BV method could effectively remove inhibitors of infection existing in the culture fluid of a retroviral producer line, and have enhanced the gene transfer into $CD34^+$ BMCs using an amphotropic retrovirus vector (*15*).

Recently, Hennemann *et al.* claimed that gene transfer occurred efficiently on a tissue culture plate without coating with native FN or rFN-CH-296 (*17, 20*), and that a positively charged surface, like that of a tissue culture plate, could capture a retrovirus vector on its surface as effectively as native FN (*20*). Although they only used native FN in their experiments, they claimed that rFN-CH-296 did not work, assuming that native FN and rFN-CH-296

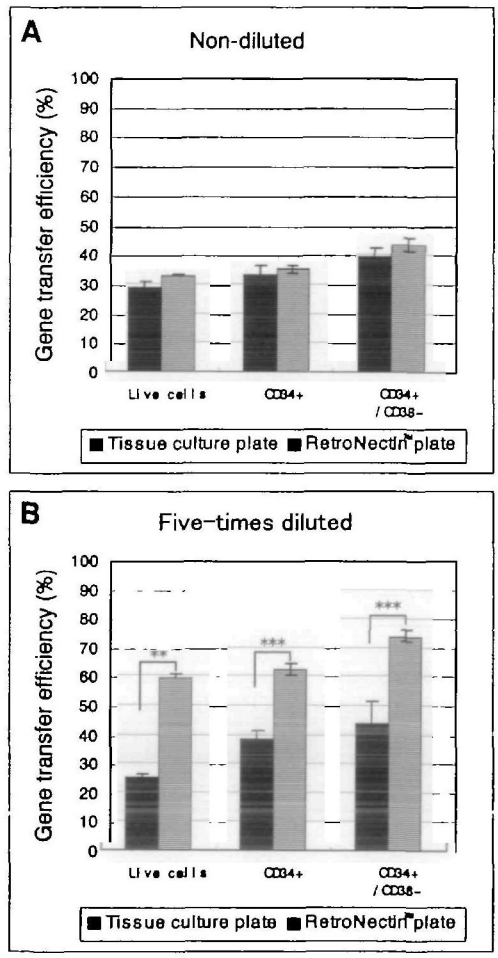

Fig. 1. **Analysis of gene transfer efficiency by BV+SN infection.** Purified $CD34^+CD38^-$ bone marrow cells were prestimulated with FL + SCF + IL-3 + IL-6 + sIL-6R for 2 days and then infected with non-diluted (A) or 5-times diluted GALV retrovirus SN (B). Stimulated $CD34^+CD38^-$ cells were suspended in viral SN and then infected in a tissue culture plate or RetroNectin™ plate to which virus had previously been bound (BV + SN infection). Two days after the infection, GFP^+ total live cells, GFP^+ $CD34^+$ cells, and GFP^+ $CD34^+CD38^-$ cells were assessed by FACS. Black bars represent the gene transfer efficiency on tissue culture plates (Hennemann's protocol). Hatched bars represent the gene transfer efficiency on RetroNectin™ plates. Values shown are means ± SD. ** $p < 0.005$, *** $p < 0.001$.

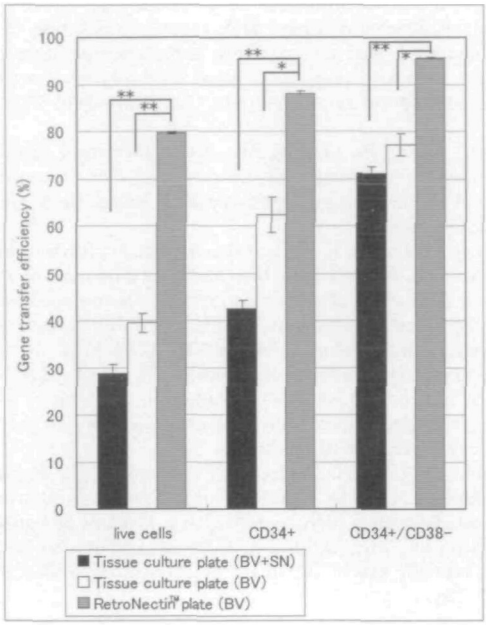

Fig. 2. **Analysis of gene transfer efficiency by BV infection.** Non-diluted GALV retrovirus SN was bound to a tissue culture plate or a RetroNectin™ plate. After binding, the SN was discarded and then 2-day-prestimulated $CD34^+CD38^-$ BMCs were placed in the wells of both plates in serum-free medium supplemented with FL + SCF + IL-3 + IL-6 + sIL-6R. Hennemann's protocol was performed in parallel. Two days after the infection, GFP^+ total live cells, GFP^+ $CD34^+$ cells, and GFP^+ $CD34^+CD38^-$ cells were assessed by FACS. Black bars represent the gene transfer efficiency with Hennemann's protocol (BV + SN infection). Open bars represent the gene transfer efficiency with BV infection on a tissue culture plate. Hatched bars represent the gene transfer efficiency with BV infection on a RetroNectin™ plate. Values shown are means ± SD. * $p < 0.05$, ** $p < 0.005$.

work equally well. However, it has already been well documented by us and other research groups that the native FN does not help as to the transduction efficiency of $CD34^+$ BMCs (*21, 22*). The CS-1 sequence of IIICS, the VLA-4 ligand, is completely buried within the folded conformation of native FN. The proteolytic digestion of native FN with some selected proteases induced the expression of the CS-1 sequence (*23*). Proteolytic fragment FN30/35 showed higher retroviral gene transfer efficiency than intact FN (*22*), maybe because of the efficient exposure of the CS-1 sequence on the surface of its molecule. Recombinant fragment FN-CH-296, which also showed higher performance as to retroviral gene transfer, is superior to full-length FN and many protocols involving rFN-CH-296 have been reported (*24–26*). In addition, most protocols involving rFN-CH-296 reported by many other researchers do not include bound virus infection, although the heparin-binding domain of FN is essential for retrovirus binding on its molecule (*12, 27, 28*). Here, in the case of the GALV pseudotyped retrovirus vector, our data showed that the efficiency of gene transfer into $CD34^+CD38^-$ BMCs on a tissue culture plate was adequately satisfactory, as Hennemann *et al.* indicated (*17*), but we demonstrated that a RetroNectin™ plate could capture the GALV vector more strongly on the rFN-CH-296 molecule and that the RetroNectin™ BV method could remove inhibitors of infection in a viral supernatant more effectively, and allow more efficient gene transfer through the co-localization of target BMCs and the GALV vector. Thus, this RetroNectin™ BV method would provide the most useful protocol including clinical trials for introducing genes into $CD34^+CD38^-$ BMCs.

REFERENCES

1. Miller, A. (1992) Retroviral vectors. Curr. Top. *Microbiol. Immunol.* **158**, 1–24
2. Vile, R.G. and Russell, S.J. (1995) Retroviruses as vectors. *Br. Med. Bull.* **51**, 12–30
3. Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* **90**, 8392–8396
4. Yoshimatsu, T., Tamura, M., Kuriyama, S., and Ikenaka, K. (1998) Improvement of retroviral packaging cell lines by introducing the polyomavirus early region. *Hum. Gene Ther.* **9**, 161–172
5. Naviaux, R.K., Costanzi, E., Haas, M., and Verma, I.M. (1996) The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* **70**, 5701–5705
6. Yang, S., Delgado, R., King, S.R., Woffendin, C., Barker, C.S., Yang, Z.Y., Xu, L., Nolan, G.P., and Nabel, G.J. (1999) Generation of retroviral vector for clinical studies using transient transfection. *Hum. Gene Ther.* **10**, 123–132
7. Ledoux, J.M., Morgan, J.R., Snow, R.G., and Yarmush, M.L. (1996) Proteoglycans secreted by packaging cell lines inhibit retrovirus infection. *J. Virol.* **70**, 6468–6473
8. Forestell, S.P., Bohnlein, E., and Rigg, R.J. (1995) Retroviral end-point titer is not predictive of gene transfer efficiency: implications for vector production. *Gene Ther.* **2**, 723–730
9. Davis, J.L., Witt, R.M., Gross, P.R., Hokanson, C.A., Jungles, S., Cohen, L.K., Danos, O., and Spratt, S.K. (1997) Retroviral particles produced from human-derived packaging cell line transduce target cells with very high efficiencies. *Hum. Gene Ther.* **8**, 1459–1467
10. Seppen, J., Kimmel, R.J., and Osborne, W.R.A. (1997) Serum-free production, concentration and purification of recombinant retroviruses. *BioTechniques* **23**, 788–790
11. Bowles, N.E., Eisensmith, R.C., Mohuiddin, R., Pyron, M., and Woo, S.L.C. (1996) A simple and efficient method for the concentration and purification of recombinant retrovirus for increased hepatocyte transduction *in vivo*. *Hum. Gene Ther.* **7**, 1735–1742
12. Hanenberg, H., Xiao, X.L., Dilloo, D., Hashino, K., Kato, I., and Williams, D.A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nat. Med.* **2**, 876–882
13. Kimizuka, F., Taguchi, Y., Ohdate, Y., Kawase, Y., Shimojo, T., Hashino, K., Kato, I., Sekiguchi, K., and Titani, K. (1991) Production and characterization of functional domains of human fibronectin expressed in *Escherichia coli*. *J. Biochem.* **110**, 284–291
14. Chono, H., Morishita, M., Hashino, K., Asada, K., Fujinaga, K., and Kato, I. (1999) Efficient gene transfer method: combination of low-speed centrifugation and fibronectin assisted retrovirus gene transfer [abstract]. The 5th Annual Meeting, 1999, of the Japan Society of Gene Therapy
15. Chono, H., Konishi, H., Hashino, K., Morishita, M., Ueno, M., Asada, K., and Kato, I. (2000) Efficient retrovirus-mediated gene transfer into $CD34^+$ bone marrow stem and progenitor cells from human by the infection method with RetroNectin™-bound virus [abstract]. The 6th Annual Meeting, 2000, of the Japan Society of Gene Therapy
16. Miller, A.D., Garcia, J.V., von Suhr, N., Lynch, C.M., Wilson, C., and Eiden, M.V. (1991) Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* **65**, 2220–2224
17. Hennemann, B., Oh, I.H., Chuo, J.Y., Kalberer, C.P., Schley,

P.D., Rose-John, S., Humphries, R.K., and Eaves, C.J. (2000) Efficient retrovirus-mediated gene transfer to transplantable human bone marrow cells in the absence of fibronectin. *Blood* **96**, 2432–2439
18. Fischer, M., Goldschmitt, J., Peschel, C., Brakenhoff, J.P., Kallen, K.J., Wollmer, A., Grotzinger, J., and Rose-John, S. (1997) A bioactive designer cytokine for human hematopoietic progenitor cell expansion. *Nat. Biotechnol.* **15**, 142–145
19. Tajima, S., Tsuji, K., Ebihara, Y., Sui, X., Tanaka, R., Muraoka, K., Yoshida, M., Yamada, K., Yasukawa, K., Taga, T., Kishimoto, T., and Nakahata, T. (1996) Analysis of interleukin 6 receptor and gp130 expressions and proliferative capability of human $CD34^+$ cells. *J. Exp. Med.* **184**, 1357–1364
20. Hennemann, B., Chuo, J.Y., Schley, P.D., Lambie, K., Humphries, R.K., and Eaves, C.J. (2000) High-efficiency retroviral transduction of mammalian cells on positively charged surfaces. *Hum. Gene Ther.* **11**, 43–51
21. Williams, D.A., Rios, M., Stephens, C., and Patel, V.P. (1991) Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. *Nature* **352**, 438–441
22. Moritz, T., Patel, V.P., and Williams, D.A. (1994) Bone marrow extracellular matrix molecules improved gene transfer into human hematopoietic cells via retroviral vectors. *J. Clin. Invest.* **93**, 1451–1457
23. Ugarova, T.P., Ljubimov, A.V., Deng, L., and Plow, E.F. (1996) Proteolysis regulates exposure of the IIICS-1 adhesive sequence in plasma fibronectin. *Biochemistry* **35**, 10913–10921
24. Pollok, K.E. and Williams, D.A. (1999) Facilitation of retrovirus-mediated gene transfer into hematopoietic stem and progenitor cells and peripheral blood T-lymphocytes utilizing recombinant fibronectin fragments. *Curr. Opin. Mol. Ther.* **1**, 595–604
25. Sanyal, A. and Schuening, F.G. (1999) Increased gene transfer into human cord blood cells by centrifugation-enhanced transduction in fibronectin fragment-coated tubes. *Hum. Gene Ther.* **10**, 2859–2868
26. Murray, L., Luens, K., Tushinski, R., Jin, L., Burton, M., Chen, J., Forestell, S., and Hill, B. (1999) Optimization of retroviral gene transduction of mobilized primitive hematopoietic progenitors by using thrombopoietin, Flt3, and Kit ligands and RetroNectin culture. *Hum. Gene Ther.* **10**, 1743–1752
27. Hanenberg, H., Hashino, K., Konishi, H., Hock, R.A., Kato, I., and Williams, D.A. (1997) Optimization of fibronectin-assisted retroviral gene transfer into human $CD34^+$ hematopoietic cells. *Hum. Gene Ther.* **8**, 2193–2206
28. Asada, K., Uemori, T., Ueno, T., Hashino, K., Koyama, N., Kawamura, A., Kato, I. (1998) Enhancement of retroviral gene transduction on a dish coated with a cocktail of two different polypeptides: one exhibiting binding activity toward target cells, and the other toward retroviral vectors. *J. Biochem.* **123**, 1041–1047