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RECOMBINANT GENE EXPRESSION IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS TRANSDUCED BY RETROVIRAL VECTORS

James A. Zwiebel,[1] Scott M. Freeman,[2,4] Kenneth Cornetta,[2,5] Reza Forough,[3] Thomas Maciag,[3] and W. French Anderson[2]

[1]Lombardi Cancer Center, Georgetown Unversity, Washington, DC 20007

[2]Molecular Hematology Branch, National Heart, Lung, and Blood Institute, Bethesda, MD 20893

[3]Laboratory of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences American Red Cross Rockville, MD 20855

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Summary: Endothelial cells are attractive targets for gene transfer because of their immediate contact with the bloodstream, and, therefore, they might serve as vehicles for therapeutic drug delivery. Recently, we and others reported that endothelial cells of animal origin efficiently express both secretory and nonsecretory recombinant proteins (1-4). We now show that human endothelial cells are also capable of expressing a recombinant gene following transduction with retroviral vectors. Human umbilical vein endothelial cells were transduced with either the N2 or the SAX vector. Following selection with G418, cells transduced by both vectors were found to express neophosphotransferase activity, the product of the neomycin resistance gene. The fact that a recombinant gene can be readily inserted and efficiently expressed into human endothelial cells suggests that these cells may be able to serve a role in human gene therapy.

Genetically engineered endothelial cells, by virtue of their direct contact with the circulating blood, would be ideally situated for therapeutic protein delivery. There are a number of reports of succesful gene transfer and expression in nonhuman

[4]Current address: University of Rochester, Rochester, NY 14642, and Metabolism Branch, National Cancer Institute, Bethesda, MD 20892.

[5]Current address: Department of Medicine, University of Wisconsin, Madison, WI 53714.

ABBREVIATIONS: ADA, adenosine deaminase; ECGF, endothelial cell growth factor; HPLC, high performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; LTR, long terminal repeat; Neo^R, neomycin resistance; NPT, neophosphotransferase; SV40, simian virus 40.

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endothelial cells (1-4). In the present study we demonstrate the feasibility of recombinant gene transfer and expression in endothelial cells of human origin. Amphotropically packaged N2 and SAX retroviral vectors were able to transduce human umbilical vein endothelial cells. Cells that survived selection in G418 contained abundant neophosphotransferase activity.

MATERIALS AND METHODS

Cells. HUVEC (a gift from M. Gibrone) were were grown on fibronectin-coated dishes (3.5 ug/cm^2) in growth medium consisting of Medium 199 with 10% fetal calf serum, 5 U./ml heparin, and 10 ng/ml HPLC-purified ECGF. HUVEC identity was confirmed by their expression of von Willebrand protein (5) and the presence of human alpha satellite DNA sequences (6). The preparation of ECGF has already been described (7).

Vector transduction. The PA317 amphotropic packaging cell line (8), obtained from R. Dusty Miller (Fred Hutchinson Cancer Institute, Seattle, WA), was used to generate viral stocks of the N2 (9) and the SAX (10) retroviral vectors. Supernatants containing $>10^6$ G418-resistant colony forming units/ml were used to transduce HUVEC as follows: 24 hours prior to infection HUVEC were plated at a 1:2 split ratio onto fibronectin-coated 100 mm tissue culture dishes. The HUVEC were incubated with 5 ml of N2-containing supernatant for 2-4 hours at 37°C. Five ml of HUVEC growth medium was added and the cells were allowed to incubate overnight before the culture medium was replaced. Twenty four hours later the HUVEC were placed in G418 (Geneticin, GIBCO) selection. All procedures involving infectious viral particles were carried out in accordance with biosafety level 2 practices (11).

NPT assay. The in situ assay for detection of the neo^R gene protein product was performed as described (12) by measuring the catalyzed transfer of a γ-^{32}P from ATP to kanamycin over the region of NPT activity in an SDS-polyacrylamide gel.

Southern hybridization. Cellular DNA was prepared (13), then digested at 37°C overnight with 40-50 units of the indicated restriction enzyme in buffers provided by the manufacturer (BRL and Boehringer-Mannheim). DNA transfer and hybridization were carried out according to established procedures (14). The DNA digests were separated on a 1% agarose gel and transferred to a 0.45 μm nylon membrane (Nytran, Schleicher & Schuell) by capillary action. The membrane was allowed to hybridize overnight with a 1.5 kilobase (kb) ^{32}P-labelled neomycin resistance (neo^R) gene probe obtained by Eco RI digestion of the N2 vector. Final wash was carried out for one hour at 68°C in 0.1XSSC, 0.1% SDS.

RESULTS

The HUVEC survival curve in the presence of G418 is shown in Figure 1. G418 at a concentration of 100 ug/ml of active compound was sufficient to completely kill HUVEC in culture. Selection of transduced HUVEC were carried out at this concentration. For comparison, the same G418 preparation killed all NIH-3T3 cells at a concentration of 400 ug/ml.

The presence of vector DNA in transduced HUVEC was confirmed by Southern hybridization. There is one Sac I site in each of the long terminal repeat

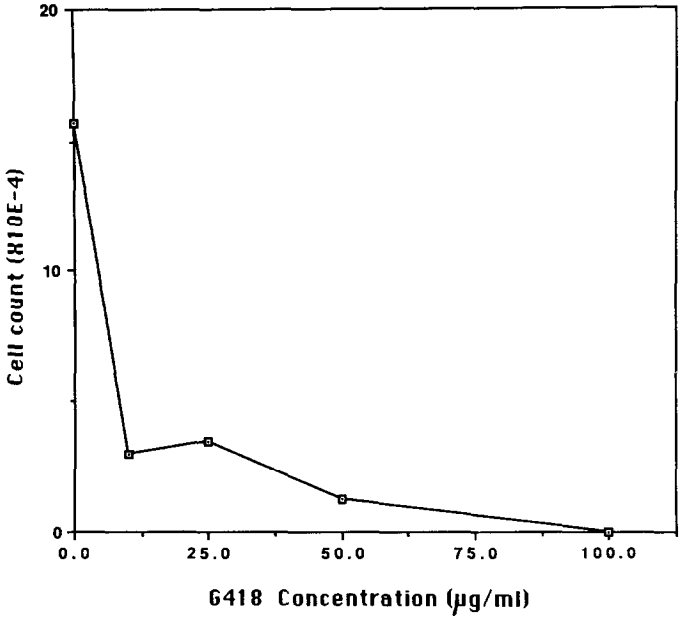

Figure 1. HUVEC G418 sensitivity. HUVEC (passage 7) were plated onto fibronectin-coated 6-well dishes (Costar) at a seeding density of 10^5 cells per well. After overnight incubation, G418 was added at the indicated concentrations of active compound. Cells were fed every other day with G418-containing growth medium. Cells were counted 7 days after plating.

(LTR) sequences that flank the SAX vector. The predicted 4.9 kb fragment was detected following Sac I digestion of SAX-transduced HUVEC (Figure 2, lane 3). The band density suggested that, on average, two to three copies of the provirus were present in each cell. Because there are no Sac II recognition sites within the SAX

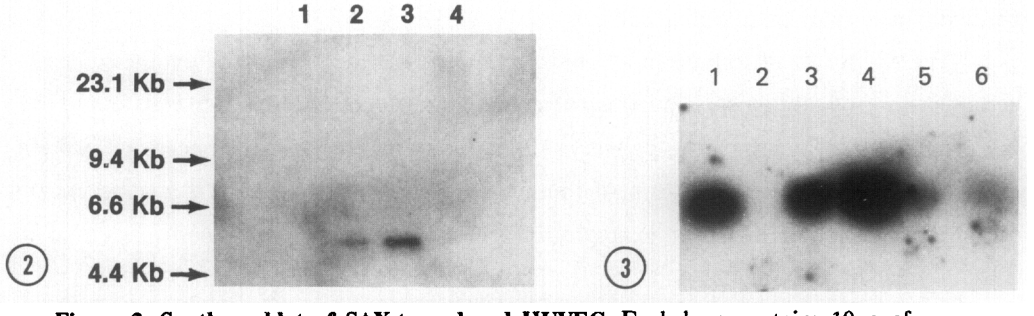

Figure 2. Southern blot of SAX-transduced HUVEC. Each lane contains 10µg of cellular DNA; **lane 1**: uninfected HUVEC; **lane 2**: uninfected HUVEC mixed with 10 pg Sac I-digested SAX plasmid (equivalent to approximately one copy of vector DNA per cell); **lane 3**: Sac I digest of SAX-transduced HUVEC; **lane 4**: Sac II digest of SAX-transduced HUVEC.

Figure 3. In situ NPT assay. Each lane contains 10^5 cell equivalents of freeze-thawed cell lysates; **lane 1**: mouse fibroblasts expressing the neo^R gene; **lane 2**: no cells; **lanes 3 & 4**: N2-transduced HUVEC; **lanes 5 & 6**: SAX-transduced HUVEC. Duplicate lanes contain cell lysates from separate dishes.

vector, digestion with that restriction enzyme gives an indication of the number of proviral integration sites within the cell population. The faint smear that is visible (Figure 2, lane 4) suggests that multiple integrations in fact have occurred.

The retroviral vector N2 has the neo^R gene under the transcriptional control of the Moloney murine leukemia virus LTR promoter. SAX contains an SV40-promoted human ADA cDNA in addition to the neo^R gene. HUVEC transduced with these vectors were selected in G418 and analyzed for NPT expression (Figure 3). Both N2 (lanes 3 & 4) and SAX-transduced (lanes 5 & 6) HUVEC expressed the neo^R gene product, NPT. HUVEC transduced with the Moloney LTR-promoted neo^R gene (N2) contained a relatively greater amount of NPT activity than cells transduced with the SV40-promoted neo^R gene (SAX). This suggests that the Moloney LTR is functioning more efficiently in these cells.

DISCUSSION

The studies reported here demonstrate that new genetic material can be successfully introduced into endothelial cells of human origin using high titer recombinant retroviral vectors. The high vector copy number found to be present in the SAX-transduced HUVEC may be explained by replication-competent helper virus that was present (Unpublished observation). This may have resulted in rescue of the SAX provirus and reinfection of the cells in culture. While we were able to demonstrate transduction of HUVEC with the ADA-containing SAX vector, recombinant ADA expression could not be assessed: the cells used in this study already have normal levels of endogenous ADA. Consequently, we evaluated vector function instead by assaying NPT activity. Studies with ADA-deficient endothelial cells will be needed to be performed in order to determine how well recombinant ADA is expressed in human endothelial cells.

The ability to introduce functional recombinant genes into endothelial cells should have applicability in a number of areas. Transduced endothelial cells could play a role in therapeutic drug delivery, either by the secretion of a necessary protein into the bloodstream or as a form of immobilized enzyme therapy with a nonsecretory recombinant gene product. For example, in severe combined immunodeficiency due to ADA deficiency, endothelial cells expressing a functional ADA gene may be able to metabolize circulating adenosine and deoxyadenosine, the toxic substances that are responsible for the loss of T and B lymphocytes. Drug delivery by genetically engineered endothelial cells could be designed to be local or systemic. While endothelial cells already play an important role in hemostasis (15), these cells could be modified to secrete coagulation proteins for either local effects, such as with a plasminogen activator in order to maintain prosthetic graft patency, or for systemic

delivery, such as Factor VIII for hemophilia. Another use for genetically marked endothelium might be for the evaluation of new seeding methods and materials for vascular prostheses (16, 17). Finally, endothelial cells have many important functions relating to coagulation and inflammation, and the effects of retroviral transduction upon these functions will need to be assessed.

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REFERENCES

1. Zwiebel, J.A., Freeman, S. M., Kantoff, P.W., Cornetta, K., Ryan, U.S., and Anderson, W.F. (1989) Science 243, 220-222.
2. Nabel, E.G., Plautz, G., Boyce, F.M., Stanley, J.C., Nabel, G.J. (1989) Science 244, 1342-1344.
3. Wilson, J.M., Birinyi, L.K., Salomon, R.N., Libby, P., Callow, A.D., and Mulligan, R.C. (1989) Science 244, 1344-1346.
4. Dichek, D., Neville, R., Zwiebel, J.A., Freeman, S.M., Leon, M.B., and Anderson, W.F. (1989) Circulation 80, 1347-1353.
5. Jaffe, E.A., Hoyer, L.W., and Nachman, R.L. (1973). J. Clin. Invest., 52, 2757.
6. Waye, J.S., and Willard, H.F. (1986). Mol. Cell. Biol. 6, 3156-3165.
7. Burgess, W.H., Mehlman, T., Friesel. R., Johnson, W.V., and Maciag, T. (1985) J. Biol. Chem. 260, 11389-11392.
8. Miller, A.D. and Buttimore, C. (1986) Mol. Cell. Biol. 6, 2895-2902.
9. Armentano, D., Yu, S.-F., Kantoff, P.W., von Ruden, T., Anderson, W.F., and Gilboa, E. (1987) J. Virol. 61, 1647-1650.
10. Kantoff, P.W., Kohn, D.B., Mitsuya, H., Armentano, D., Sieberg, M., Zwiebel, J.A., Eglitis, M.A., McLachlin, J.R., Wiginton, D.A., Hutton, J.J., Horowitz, S.D., Gilboa, E., Blaese, R.M., and Anderson, W.F. (1986) Proc. Natl. Acad. Sci. USA 83, 6563-6567.
11. Centers for Disease Control (1988) MMWR 37, Supp. No. S-4, 6-8.
12. Eglitis, M.A., Kantoff, P., Gilboa, E., and Anderson, W.F. (1985) Science 230 (4732), 1395-1398.
13. Gross-Bellard, M., Oudet, P., and Chambon, P. (1973) Eur. J. Biochem. 36, 32-38.
14. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
15. Jaffe, E.A., (1990) In Hematology (W.J. Willaims, E. Beutler, A.J. Erslev, and M.A. Lichtman, Ed.) pp. 1322-1337. McGraw-Hill Book Company, New York.
16. Herring, M., and Glover, J.L., Editors (1987). Endothelial Seeding in Vascular Surgery. Grune & Stratton, Inc., Orlando, FL.
17. Zilla, P.P. Fasol, R.D., and Deutsch, M., Editors. (1987) Endothelialization of Vascular Grafts, 1st European Workshop on Advanced Technologies in Vascular Surgery, Vienna, November 5-6, 1986. Karger, Basel.