A VSV-G Pseudotyped HIV Vector Mediates Efficient Transduction of Human Pulmonary Artery Smooth Muscle Cells

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Received June 29, 2000; in revised form, August 23, 2000. Accepted September 11, 2000

Abstract: Attempts were made to infect human vascular smooth muscle cells derived from the pulmonary artery (hPASMC) with two different human immunodeficiency virus (HIV) vector systems. ADA/Luc or HXB2/Luc were generated by cotransfection of luciferase reporter gene vector, pNL4-3-Luc-E-R', and one of two envelope expressing vectors, pSMADA (R5) or pSMHXB2 (X4). The VSV-G/Luc or VSV-G/GFP were produced by a three-plasmid expression system which consisted of vesicular stomatitis virus G protein (VSV-G) expressing vector, packaging plasmid, and one of two reporter genes (pHR'-CMV-Luc or pHR'-CMV-GFP). We used hPASMC, U87.CD4.CCR5 and U87.CD4.CXCR4 for infection. Neither ADA/Luc nor HXB2/Luc could infect hPASMC, though they could infect U87.CD4 with corresponding coreceptors. On the other hand, the transduction of both VSV-G/Luc and VSV-G/GFP to hPASMC was remarkable. At day 3, the relative proportion of positive cells of hPASMC infected with VSV-G/GFP was 15%. The above finding indicates a direct role of HIV-1 infection in pulmonary hypertension ‘a rare complication of HIV-1 infection’ and HIV-based vectors could introduce foreign genes into hPASMC for gene therapy of pulmonary hypertension.

Key words: Vector, Smooth muscle cell, Pulmonary hypertension, HIV

Acquired immunodeficiency syndrome (AIDS) is associated with numerous cardiopulmonary complications. Pulmonary hypertension (PH) is a well accepted, although rare, complication associated with HIV infection (18, 28). The incidence of PH in HIV-positive individuals may be as high as 0.5% (29). But, the pathogenesis of the association of PH in HIV infection has not been clarified yet. Some reports have shown that the route of infection is not related to the association (11). It has also been described that no correlation existed between either CD4 counts or a history of pulmonary infection and the development of PH. The one-year survival rate for patients with PH and HIV was 51%, while it was 68% for patients with primary pulmonary hypertension (PPH) (21). A beneficial effect on the pressure gradient by antiretroviral treatment was also reported (25). However, it remains unclear whether HIV infection itself causes a secondary form of PH or whether an increased incidence of PPH is observed in HIV-infected patients. It is difficult to demonstrate virus sequences in muscle cells and endothelial cells in PH (22), however the association of PH with virus infection has been demonstrated in animal models. Pulmonary arteriopathy with initial proliferation in macaques experimentally infected with the simian immunodeficiency virus and pulmonary hypertension in a murine model of acquired immunodeficiency syndrome has also been reported (10, 20). Progressive focal to segmental thickening of the intima and media of the artery was demonstrated in macaque monkeys infected with simian immunodeficiency virus (20). Medial hypertrophy of the small pulmonary arteries was also demonstrated in HIV-infected patients with PH (14). It has been suggested that the development of PH in HIV infection is mediated by the HIV-1 induced release of inflammatory mediators such as vascular endothelial growth factors (VEGF) or platelet derived growth factors (2, 15). Released virus

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Abbreviations: CCR5, CC-chemokine receptor 5; CXCR4, CXC-chemokine receptor 4; HTLV-1, human T-cell leukemia virus type 1; IL-2, interleukin 2.
proteins such as gp120 or tat may also contribute to the development of vascular disease in HIV infected individuals (1, 17). In this paper, we attempted to infect human smooth muscle cells derived from pulmonary artery with different HIV-1 vectors to verify whether a direct virus infection of hPASMC could play a role in PH, and tried to transduce foreign genes to the cells using the HIV vectors.

Materials and Methods

Plasmids. pSMHXB2 (32) and pSMADA (8, 33), expressing the HIV envelope gene of the X4 strain HXB2 and R5 strain ADA, were kindly provided by Dr. C. Weiss (CBER, FDA). P. pyralis luciferase expression vector pN14-3-Luc-E' R' was from Dr. D. Littman (Skirball Institute, New York University, N.Y., U.S.A.) (6, 19). The HIV lentiviral vector system including three expression plasmids, vesicular stomatitis virus G protein (VSV-G) expressing vector pMD.G, packaging plasmid pCMVA8.2, expression cassettes for luciferase (lac) pHRCMV-Luc or green fluorescent protein (GFP) pHRCMV-GFP were provided by Dr. Inder Verma (24).

Cell culture. All cells were cultured in 100% humidified air at 37 C with 5% CO2. A human embryonic kidney cell line, 293T (26), and human astroglia cell lines U87, expressing CD4 antigen and coreceptor CXCR4 (U87.CD4.CXCR4), and CCR5 (U87.CD4.CCR5) (8) were provided by Dr. D. Littman. The cells were grown in Dulbecco’s modified Eagle’s medium (D-MEM) with high glucose (GibcoBRL, Grand Island, N.Y., U.S.A.) containing 10% fetal calf serum (FCS) (D-MEM complete medium). G418 (Geneticin Disulfate, Wako Pure Chem., Osaka, Japan), 300 µg/ml, and 1 µg/ml puromycin (Sigma, St. Louis, Mo., U.S.A.) were added as selection reagents for U87.CD4 with coreceptors. An IL-2 independent double-negative (CD4+CD8-) HTLV-1 infected T cell line, 43Ti, established from a patient with double-negative adult T cell leukemia (ATL) (13), was provided by Dr. Maeda of Kyoto University. The cells were cultured in RPMI 1640 (GibcoBRL) supplemented with 10% FCS in the presence of 100 µg/ml kanamycin. Monocyte-derived macrophages (MDM) were obtained as previously described (16) with slight modifications. Briefly, monocytes were enriched from peripheral blood mononuclear cells (PBMC) of healthy HIV-1 seronegative donors through Ficoll-PaqueTM Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and incubated at 37 C for 24 hr in a plastic dish coated with human AB serum. One day later, nonadherent cells were removed, and the adherent cells were cultured for 5 days in RPMI 1640 (GibcoBRL) supplemented with 10% FCS, and 5% giant cell tumor-conditioned medium (Igen, Inc., Rockville, Md., U.S.A.). Adherent macrophages were treated with 0.5 mm EDTA in phosphate-buffered saline (PBS) for 10 min to harvest the cells. Human smooth muscle cells from normal tissue of the pulmonary artery (hPASMC) (Clonetics Co., San Diego, Calif., U.S.A.) were cultured according to the manufacturer’s instructions (3). hPASMC were cultured in smooth muscle cell growth medium (SmGM-2 BulletKit) supplemented with 0.5 ng/ml human recombinant epidermal growth factor, 5 µg/ml insulin, 2 ng/ml human recombinant fibroblast growth factor, 50 µg/ml gentamicin, 50 ng/ml amphotericin-B and 5% FCS. The culture medium was changed every 3–4 days, and the cells at passages 4–6 were used for the experiments. The cell viability was counted by the trypan blue dye exclusion method, and the viability of cells used for the experiments always exceeded 95%.

Production of the vectors. ADA/Luc and HXB2/Luc were generated by cotransfection of the pNL4-3-Luc-E' R' and one of the Env-expressing vectors, pSMADA or pSMHXB2, into 293T cells upon lipofectamine-plusTM following the manufacturer’s instructions (GibcoBRL). The VSV-G pseudotyped HIV vector containing the luciferase (VSV-G/Luc) or GFP reporter gene (VSV-G/GFP) were obtained by cotransfection of three plasmids, pMD.G, pCMVA8.2 and pHRC'MV-Luc or pHRC'MV-GFP into 293T cells. Two to three days after cotransfection the supernatants were cleared by low-speed centrifugation followed by filtration, and the p24 antigen of HIV-1 was determined by an antigen capture assay (Abbott Laboratories, Abbott Park, Ill., U.S.A.). The amounts of viruses were expressed as the concentration of p24 antigen.

Infection and luciferase assay. One day before infection, U87.CD4 with coreceptors (4 x 10^4), hPASMC (4 x 10^4) or MDM (2 x 10^6) were plated into each well of 24-well microplates in 500 µl of the corresponding medium. Subsequently, the cells were infected with various amounts (2.5–160 ng) of the viruses at a total volume of 500 µl. For the infection of 43Ti cells, 1 x 10^6 cells were infected in 15 ml tubes at 37 C for 3 hr at a total volume of 1 ml. After adding 9 ml of fresh medium, the cells were cultured for 3 days. The amount of each pseudotyped virus is indicated in the figure legend. At the indicated days, the infected cells were washed with PBS three times and lysed in 100 µl of luciferase lysis buffer (Promega, Madison, Wisc., U.S.A.). The luciferase activity in 20 µl of lysate was measured with a luminometer (1251 Bio Orbit, Turku, Finland). The luciferase activity from non-infected cells was used as a negative control.

Infection and GFP expression. hPASMC and MDM, 1 x 10^6 cells/well, were infected with different doses of
HIV vector VSV-G/GFP (1 ml) in 6-well plates, and 43Ti cells were infected as described above. To quantify the GFP-positive cells, VSV-G/GFP infected target cells were investigated using flow cytometry (FACSscan, Becton Dickinson Immunocytometry System, U.S.A.). MDM and hPASMC were harvested using a cell scraper after 5-min incubation in EDTA-PBS. 43Ti cells were collected by centrifugation. The recovered cells were washed twice with PBS containing 0.5% BSA and 0.01% sodium azide (PBS-BSA) and resuspended at a concentration of 1 x 10^6/ml with PBS-BSA. GFP expression in VSV-G/GFP infected hPASMC was observed under a laser scanning confocal imaging system (Micro Radiance, Bio-Rad, Tokyo) and the positive cell percent was counted under an inverted fluorescent microscope (Axiovert S100 equipped with a MC80 DX Microscope Camera) (Zeiss, Jena, Germany).

Results

Production of Pseudoviruses

After cotransfection of corresponding plasmids into 293T cells, the harvested media from the various transfectants were subjected to p24 antigen assay as described in "Materials and Methods." The p24 antigen amounts of HXB2/Luc, ADA/Luc, VSV-G/Luc and VSV-G/GFP were as high as 694±24, 383±23, 421±17 and 478±29 ng/ml, respectively.

Infection of the Cells with Pseudotyped Viruses

hPASMC cells derived from smooth muscle cells of the pulmonary artery were used as target cells because these cells are reported to be responsible for the development of PH (14, 20). U87.CD4.CXCR4 cells are known to be infected with X4 tropic virus and U87.CD4.CCR5 cells can be infected with R5 tropic virus (8). Dose-dependent increases of luciferase activities were observed in U87.CD4.CXCR4 cells infected with HXB2/Luc and in U87.CD4.CCR5 infected with ADA/Luc. Neither pseudotypes, however, could infect hPASMC cells even at 160 ng/well (Fig. 1A). These findings indicate that a direct role of HIV-1 infection of hPASMC is less likely for the development of PH. On the other hand, VSV-G/Luc could infect all of the target cells in a dose-dependent manner. The luciferase activities of hPASMC tend to be lower than U87.CD4 with coreceptors, though the viability was more than 90% after culturing (Fig. 1B).

The pathogenesis of PH is complex and released mediators from HIV-1 infected lymphocytes or macrophages might be responsible for the development of PH (2, 15, 17, 22). Therefore, We comparatively studied whether the HTLV-1 infected double-negative (CD4^-CD8^-) cells, 43Ti cells or MDM could be infected with VSV-G/Luc or ADA/Luc. The luciferase activities from 43Ti cells infected with VSV-G/Luc, and from MDM infected with ADA/Luc or VSV-G/Luc were much lower than that from hPASMC: luciferase activity (relative luminescence units, RLU) being 948, 3,853 and 662 at day 3, respectively. The MDM infected with VSV-G pseudotyped virus was monitored for 18 days (Fig. 2). The luciferase activity rose to more than 3,000 at day 7, but did not rise significantly even at day 18.

We also attempted to compare the infectivity of VSV-G vectors to various cells as described below.

![Fig. 1. Infection of different target cells with the pseudotyped viruses. VSV-G/Luc, ADA/Luc and HXB2/Luc were used to infect the different target cells including U87.CD4.CXCR4, U87.CD4.CCR5 and hPASMC. The mean of triplicate experiments with representative data of three different experiments is shown, SD was less than 5%. The luciferase activities (RLU) of virus-negative cultures were less than 100 in all of the experiments. A: Dose-dependent infection of ADA/Luc or HXB2/Luc to the U87.CD4 cells with corresponding coreceptor or hPASMC. □, U87.CD4.CXCR4 infected with HXB2/Luc; △, U87.CD4.CCR5 infected with ADA/Luc; ■, hPASMC infected with HXB2/Luc; ▲, hPASMC infected with ADA/Luc. B: Dose-dependent infection of VSV-G/Luc to different target cells. ●, hPASMC; △, U87.CD4.CCR5; □, U87.CD4.CXCR4.](image-url)
Representative doses of VSV-G pseudotype virus containing the GFP reporter gene (VSV-G/GFP) were used to infect hPASMC (1 × 10^5 cells), 43Ti (1 × 10^6) and MDM (1 × 10^5 cells). After 3 days post-infection, the target cells were collected and the GFP intensity was measured by flow cytometry. The relative proportion of positive cells of hPASMC increased in a dose-dependent manner and 15% of the cells expressed GFP at the highest concentration of VSV-G/GFP, but approximately 2% of the cells were positive when MDM and 43Ti were used (Fig. 3). The number of positive cells in MDM and 43Ti did not increase even 18 days after infection (data not shown). The GFP expression in hPASMC was easily detected by a fluorescent microscope and the positive numbers were essentially the same as flow cytometry analysis. The percentages of positive cells increased in a dose-dependent manner and 21% of the cells expressed GFP at the highest concentrations (Fig. 4, A and B). These findings suggest that significant numbers of hPASMC could express the desired genes with the HIV-1 lentivirus vector used here.

**Discussion**

In this paper, we investigated whether hPASMC could be infected with two pseudotyped viruses, HXB2/Luc and ADA/Luc, bearing representative envelopes of HIV-1. The luciferase activities of the pseudotype virus-infected cells were very low, though the viruses successfully infected U87.CD4 with corresponding coreceptors. As the reporter gene is the luciferase gene, it makes the method very sensitive for detecting virus entry (8). The lack of luciferase activity in hPASMC infected with HXB2/Luc or ADA/Luc strongly suggests that the cells are not permissive to X4 or R5 HIV-1 entry. Therefore, infection of smooth muscle cells by HIV-1 is not likely a cause of PH in HIV infected individuals. However, it could also be possible that a very rare HIV strain could infect the cells in vivo and cause some pathological changes in the muscle cells. These possibilities were also raised in the case of cardiomyositis, in which HIV-1 could not infect cardiomyocytes though positive signals of *in situ* hybridization could be detected (12, 27). The pseudotyped vector with the envelope gene of VSV-G successfully entered into the cells and a comparable amount of synthesized reporter gene products was observed in infected hPASMC. These findings suggest that hPASMC support synthesis of the HIV proteins once they are infected with the pseudotyped viruses.

Histological studies with electron microscopy, nucleic acid analysis by *in situ* hybridization and polymerase chain reaction did not reveal direct pulmonary artery infection with the virus. However, it was clearly demonstrated that there were tubuloreticular structures in the arterial venule and microvascular endothelium. Because the structures appear to be part of the cell response to injury and have not been seen in patients with PPH (30), it appeared that HIV may have played a role in the
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The pathogenesis of these cases of PH through released mediators associated with HIV-1 infection (22). VEGF released from HIV-1 infected lymphocytes might be one source, because this factor is known to be involved in hypoxia-induced pulmonary hypertension (5, 7). The released viral proteins may also be responsible for mus-

Fig. 4. Microscopic observations of GFP expression in hPASMC. GFP expression in hPASMC cells was detected 3 days after infection with different dosages of VSV-G/GFP. The amount of cells was $1 \times 10^5$ /well in 6-well plates. A–D: Fluorescent photographs taken by a laser scanning confocal imaging system. (A, without virus; B, 10 ng/well; C, 80 ng/well; D, 160 ng/well.) The marker bar represents 100 µm. E: A photo taken by an inverted microscope ($\times 32$).
cell proliferation. Angiogenic activity of the tat protein has been reported, and this protein can bind to a specific receptor, Flk-1/KDR, on smooth muscle cells (15, 31). The envelope glycoprotein gp120 can induce the proliferation of vascular smooth muscle cells by interacting with the neuropeptide Y receptor (1).

These effects could be abrogated by the introduction of desirable genes if they could block the binding of these possible agents to the cells, interfere with the signal transduction mediated by these agents or inhibit virus replication (9, 23, 34). We have successfully infected hPASMC with VSV-G vectors. However, it is also possible that infiltrating lymphocytes that secrete cytokines or viruses play a major role in the development of PH. Therefore, we attempted to infect double-negative T cells, which do not express CD4 or CD8 antigen, with VSV-G vectors. These cells could be infected with VSV-G/Luc or GFP though the efficiencies were very low. The involvement of monocytes or macrophages in PH should also be considered because they could be reservoirs of viruses and secrete inflammatory cytokines. But our data indicated poor infection of these cells by the pseudotype viruses used here. Probably, we need to use high titers of the vectors because some researchers showed that MDM could be infected by lentivirus vectors coding the VSV-G envelope when the vectors are concentrated or the infection titer is as high as 150 ng of the p24 equivalent (24).

Very recently, smooth muscle cells transfected with nitric oxide synthetase in vitro have been found to be trapped in the lung by a natural filtering function of the pulmonary microvasculature. Such delivery could reduce the right ventricular pressure of monocrotaline-induced pulmonary hypertension. Am. J. Respir. Cell Mol. Biol. 18: 768–776.

References

with HTLV-I. Lancet **337**: 76–77.


