Transduction of Human PBMC-Derived Dendritic Cells and Macrophages by an HIV-1-Based Lentiviral Vector System

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Professional antigen-presenting cells, such as dendritic cells (DCs) and macrophages, are target cells for gene therapy of infectious disease and cancer. However, transduction of DCs and macrophages has proved difficult by most currently available gene transfer methods. Several recent studies have shown that lentiviral vector systems can efficiently transduce many nondividing and differentiated cell types. In this study, we examined the gene transfer to DCs and macrophages using a lentiviral vector system. Human DCs were propagated from the adherent fraction of peripheral blood mononuclear cells (PBMCs) by culture in medium containing GM-CSF, IL-4, and TNF-α. Human macrophages were propagated from adherent PBMCs in medium containing GM-CSF. High titers of a replication-defective vesicular stomatitis virus glycoprotein G pseudotyped HIV-1-based vector encoding the enhanced yellow fluorescent protein were produced. In immature DCs (culture days 3 and 5), transduction efficiencies of 25 to 35% were achieved at a multiplicity of infection of 100. However, the transduction efficiency was decreased in more mature DCs (culture day 8 or later). Furthermore, monocyte-derived macrophages were also transduced by the lentiviral vector system. In addition, Alu-LTR PCR demonstrated the integration of the HIV-1 provirus into the cellular genome of the transduced DCs and macrophages. Allogeneic mixed lymphocyte reactions revealed similar antigen-presenting functions of untransduced and lentivirally transduced DCs. Thus, the results of this study demonstrate that both PBMC-derived DCs and macrophages can be transduced by lentiviral vectors.

Key Words: dendritic cells (DCs); macrophages; peripheral blood mononuclear cell (PBMC); lentiviral transduction; HIV-1-based vector; vesicular stomatitis virus glycoprotein G (VSV-G); genomic integration; gene therapy.

INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC). They play a pivotal role in stimulating antigen-specific T-cells in vivo (1, 2). Because of their ability to generate a primary antitumor T-cell response, DCs are of particular interest for immunotherapeutic approaches to cancer. One strategy utilizes genetically modified DCs to establish expression of a gene that encodes a tumor-associated antigen. Although there are many gene delivery systems available, most of these vectors are not suitable for transduction of DCs. Nonviral gene transfer methods, including calcium phosphate precipitation, electroporation, and liposomal transfection, have been demonstrated to be inefficient in transducing human DCs (3). Adenoviral vectors are able to achieve high transduction rates in human DCs, but their utility may be hampered by the high multiplicity of infection (m.o.i.) required for efficient transduction and by their own inherent immunogenicity. In addition, adenoviruses are not able to integrate their genetic information into the genome of the target cells and the expression of the transferred genes remains transient (4). Retroviruses are capable of stably transducing target cells; however, cell division is required for introducing the transgene into the
host genome (5). Recently, HIV-based lentiviral vector systems have been demonstrated to transduce nondividing and terminally differentiated cell types efficiently in vitro and in vivo (6), including hematopoietic progenitor cells and neurons (7–10). Given this capacity of lentiviral vectors, we sought to transduce human peripheral blood mononuclear cell (PBMC)-derived DCs by a vesicular stomatitis virus (VSV)-G pseudotyped HIV-1-based vector system. Human monocytes/macrophages are major targets and reservoirs of human immunodeficiency viruses (11). Gene therapeutic approaches to prevent or reduce HIV replication, such as intrabody and intrakine strategies (12–14), would require delivery of genes to these cells in addition to T-cells. A promising vector system to transduce both monocytes/macrophages and T-cells is replication-defective lentiviruses. Although it has been shown that monocytic-derived macrophages can be genetically modified by lentiviral vectors (6, 15), we further examined the ability of lentiviral vectors to transduce human PBMC-derived macrophages in this study. The results demonstrate that VSV-G pseudotyped lentiviral vectors are capable of transducing human PBMC-derived DCs and macrophages. The transduction efficiencies at various m.o.i.’s of lentiviral vectors were examined.

**Material and Methods**

**Preparation of Human Macrophages and Dendritic Cells**

Human dendritic cells were prepared according to the method of Romani et al. (16) with modification (17, 18). Briefly, PBMCs were isolated by Ficoll–Hypaque gradient centrifugation (Pharmacia) of buffy coats from healthy donors, washed three times in phosphate-buffered saline (PBS), and resuspended in complete RPMI 1640 medium (10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 2.5 μg/ml amphotericin B). The cells were allowed to adhere differentially in a volume of 25 ml (2–3 × 10⁶ cells/ml) to 150-cm² plastic tissue culture flasks for 1 h at 37°C in humidified 5% CO₂. The nonadherent cells were removed by rinsing with warm PBS three times. Remaining adherent cells were harvested and cultured at a density of 1 × 10⁶ cells/ml in complete RPMI medium. For preparation of macrophages, 800 U/ml human recombinant GM-CSF (Immunex) was added to the culture medium. For propagation of DCs, 800 U/ml human recombinant GM-CSF, 500 U/ml human recombinant IL-4 (Biosource International), and 100 U/ml TNF-α (added on day 5) (R & D Systems) were used. At different times of culture, the cells were recovered by vigorous washing with 0.02% EDTA in PBS and light scraping to remove all nonadherent DCs and adherent macrophages for transduction experiments and immunophenotypic analysis.

**Plasmid Construction—Lentiviral Vector Production and Virus Titration**

The plasmid pHIV-eYFPΔenvΔvifΔvpr was generated by subcloning the enhanced yellow fluorescent protein (eYFP) reporter gene into pHIV-APΔenvΔvifΔvpr (8). pHIV-APΔenvΔvifΔvpr is derived from the HIV-1 NL-3 isolate and has a 0.62 kb deletion in Vif/Vpr, in addition to a 1.45-kb deletion in gp160 (8). The gene for the human placental alkaline phosphatase (HPAP) replaces Nef. The 0.8-kb BamHI restriction fragment of pEYFP-1 (Clontech) was used to replace the Nol-Xhol restriction fragment within pHIV-APΔenvΔvifΔvpr (which encompasses HPAP) by blunt-end ligation (T4 DNA ligase) after treatment with the Klenow fragment of Escherichia coli DNA polymerase I. The plasmid pME-CSV-G was used to pseudotype the lentiviral vector (8).

**Lentiviral Transduction of Macrophages and Dendritic Cells**

Human macrophages and DCs were transferred to 24-well tissue culture plates at a density of 0.5–1 × 10⁶ cells per well 24 h prior to transduction. Transductions were performed using m.o.i.’s ranging from 0.1 to 500 by thawing the titrated virus stocks at 37°C, mixing the appropriate volume of virus concentrate with 8 μg/ml Polybrene (Sigma), and adding the mixture to the target cells together with RPMI to achieve a total volume of 500 μl per well. After 5–6 h incubation at 37°C an additional 1 ml of complete RPMI was added. Most of the culture medium was aspirated and replaced by fresh RPMI supplemented with cytokines the day after transduction and every other day afterward. The transduced cells were examined daily using visual inspection by inverted light and fluorescence microscopy. Microscopic images were acquired using bright-field, phase-contrast, and differential-interference-contrast techniques with and without oil immersion (Axiohot, Zeiss). Photography was performed with a Hamamatsu 5380 digital camera, and images were processed using Adobe Photoshop.

**Immunofluorescent Staining and FACS Analysis**

Flow cytometric analyses were performed with a FACS Calibur apparatus and with CellQuest software (Becton–Dickinson, BD). For immunophenotypic analysis, monocytes (day 0), macrophages (day 8), and DCs (day 8) were labeled with phycoerythrin (PE)-conjugated antibodies directed against the following antigens: CD1a (BL6, Beckman Coulter), CD83 (HB15a, Beckman Coulter), CD80 (MAB104, Beckman Coulter), CD86 (2331 FUN-1, Pharmingen), HLA-DR (L243, BD), CD14 (MCP9, BD), CD3 (SK7, BD), CD19 (SJ25C1, BD), and CD56 (NKH-1, Beckman Coulter). Appropriate isotype controls were used in every experiment to determine nonspecific background staining. After suitable gating (see below), 10,000 events were collected for each marker.

The proportion of macrophages and DCs transduced by a lentiviral transfection was quantified by flow cytometry. Four to five days after transduction, the cells were recovered from the culture dishes, centrifuged at 400g for 5 min, resuspended in PBS with 2% FCS, and subjected to FACS analysis. The macrophage or DC population was identified and gated based on light-scatter properties. The fluorescent activity of gated cells was determined using histogram plots and histogram statistics.

**Alu-LTR PCR**

To demonstrate genomic integration of the lentiviral vector in the transduced DCs and macrophages, DNA was prepared from cells 30 min or 4 days after vector exposure. The cells were lysed in 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 1% Tween, and 1% NP-40 and treated with proteinase K. An Alu-LTR PCR was carried out (19) by using oligonucleotides specific for the conserved sequence of the human Alu element (5′-TCCAGGC-TACTCGGGAGGCTGAGG-3′) and for the HIV-1 LTR (5′-AGCAAGCTT-TATTGAGGCTTAAGC-3′). The samples (300 ng of genomic DNA per 50 μl reaction as template) were subjected to 5 min initial denaturation at 94°C.
and 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 66°C, 5 min extension at 72°C, and 10 min at 72°C for the final extension step (Taq-Polymerase, Promega). Following electrophoretic size fractionation on a 0.8% agarose gel and ethidium bromide staining, the PCR products were analyzed by UV imaging. A Southern hybridization was performed by using an α-32P random-labeled PCR-probe (forward primer, 5’-CACACA-CAAGGCTACTTCCCT-3’; reverse primer, 5’-GCCACTCCCCAGTCCCG-CCC-3’) corresponding to a 353-bp sequence of the U3 region within the HIV-1 LTR.

**Allogeneic Mixed Lymphocyte Reaction (MLR)**

PBMC-derived DCs from three different donors were transduced at day 3 of culture in GM-CSF and IL-4 at an m.o.i. of 100. After addition of TNF-α
at culture day 5, the cells were analyzed by FACS at day 8. DCs expressing eYFP (20–30%) were separated from untransduced cells using a Cytomation MoFlo cell sorter [forward scatter (FSC)/side scatter (SSC) and fluorescent gates were set as depicted in Fig. 2]. As determined by trypan blue staining technique, the viability of sorted DCs was higher than 90% after cell sorting.

Sorted transduced DCs and sorted untransduced DCs in comparison were irradiated with 1500 rad [137Cs] and added in graded doses to triplicate wells, each containing 1 × 10^5 allogeneic nonadherent PBMCs in 96-well tissue culture plates. Cultures were pulsed with 1 μCi of [3H]thymidine per well (5 μCi/ml [3H]TdR; New England Nuclear) during the last 18 h of 7 days coculture of stimulator and effector cells. Cells were harvested onto glass fiber filter membranes by using a Filtermate cell harvester (Packard) and tritiated thymidine incorporation was measured by scintillation counting (TopCount NXT, Packard). Wells containing only effector lymphocytes or irradiated DCs regularly incorporated less than 300 cpm [3H]TdR.

**RESULTS AND DISCUSSION**

An HIV-1 provirus with deletions in Env, Vif, and Vpr genes and with the gene for HPAP in the Nef region was constructed previously (8). HPAP was replaced by the reporter gene eYFP. In the resultant vector, HIV-eYFPΔenvΔvifΔvpr, the eYFP is under the transcriptional control of the HIV-1 LTRs without an internal promoter. High titers of VSV-G pseudotyped HIV-particles (1–5 × 10^7 IU/ml) were produced and further concentrated up to viral titers of 1 × 10^9 and 5 × 10^9 IU/ml.

Human DCs were derived from the adherent fraction of PBMCs in medium containing 800 U/ml GM-CSF and 500 U/ml IL-4 (16). TNF-α at a final concentration of 100 U/ml was added to the cultures to facilitate in vitro maturation of monocyte-derived DCs (17, 18). During the cell culture, the initially adherent or semiadherent cells became large clusters of nonadherent cells. Most of the cells showed an increasing size and developed ruffled, stellate processes of their membranes. On culture day 8, the majority of cells displayed typical DC morphology (Figs. 1A and 1C).

To characterize the PBMC-derived DCs, the cell surface molecules were monitored by flow cytometric analysis. As determined by flow cytometry, the major proportion of adherent PBMCs (80–90%) exhibited a combination of high FSC and high SSC properties, although a minor cell population (10–20%) showed a combination of low FSC and low SSC, typical of lymphocytes. In the immunophenotypic analyses the cells were gated on the population with high FSC and high SSC profiles (Fig. 2A) to determine the expression of surface markers on the cultured cells. As depicted in Fig. 3, prior to the addition of cytokines, the cells showed immunophenotypic features of monocytes: high expression of CD14 and HLA-DR and virtually no expression of CD1a. Interestingly, a small percentage of the monocyte population from different donors was repeatedly found to be positive for CD83. The immunophenotypic analyses on day 3 and day 8 of culture with GM-CSF, IL-4, and TNF-α reflected the differentiation of blood monocytes into DCs. As shown in Fig. 3, increasing levels of CD83 and CD1a were expressed on the analyzed cells during the culture, while the surface expression of CD14 decreased. The costimulatory molecules CD80 (B7.1) and CD86 (B7.2) were also markedly upregulated during the culture. These results demonstrate that adherent PBMCs differentiated into DCs under those culture conditions.

We then examined the transducibility of the cultured DCs by lentiviral vectors. The PBMC-derived cells were transduced on day 3, day 5, and day 8 of culture in GM-CSF, IL-4, and TNF-α with the lentiviral vector at various m.o.i.’s ranging from 0.1 to 500. The transduced cells were assessed daily by inverted fluorescence microscopy. Four to five days after transduction, FACS analyses were performed to measure the percentage of eYFP-ex-
pressing DCs in the cell population with high FSC and high SSC.

On day 3 and day 5 of culture, immature DCs were transduced with the lentiviral vector. At an m.o.i. of 100 an average of 23% DCs were transduced on day 3 (five repeated experiments) and on day 5 (three repeated experiments). DCs derived from different donors showed variable susceptibility to lentiviral transduction. For instance, at an m.o.i. of 300 the proportion of eYFP-expressing DCs ranged from 14 to 33% in different donors. Under the described transduction conditions, the maximum transduction efficiency of approximately 35% was

**FIG. 3.** Immunophenotyping of PBMC-derived DCs. Adherent PBMCs were analyzed by immunofluorescent staining and FACS before and during culture with GM-CSF, IL-4, and TNF-α. Results are displayed as a bar chart showing the percentages of cells (arithmetic mean, standard error) that were gated on the basis of high FSC/SSC properties (Fig. 2A) and also were positive for the listed cell surface markers (number of experiments at each time point = 3).
achieved at an m.o.i. of 100 (Fig. 4). The viability of transduced and untransduced DCs was always higher than 90% according to trypan blue exclusion and propidium iodide staining (data not shown).

To further determine that the transduced cells were DCs, we examined the culture cells under fluorescence and light microscopy with phase-contrast as well as differential-interference-contrast techniques. As shown in Fig. 1, the majority of eYFP-positive cells displayed the distinguishing features of DCs with multiple dendron-like...
processes and/or characteristic veils. We also observed the time course of transgene expression in DCs. The maximum eYFP expression in terms of fluorescence intensity and transduced cell numbers was reached on day 4 to 5 after transduction. No apparent decrease in fluorescence activity was observed even 10 days after transduction. Taken together, these results indicate that DCs can be transduced by lentiviral vector systems and that the transgene can be expressed by DCs.

The transducibility of DCs in various stages of differentiation was also compared. PBMC-derived DCs that had been cultured for 8 days in the presence of GM-CSF, IL-4, and TNF-α were markedly less transducible by VSV-G pseudotyped HIV-eYFPΔenvΔvifΔvpr virus (Fig. 4). DCs derived from eight different donors showed a similar tendency. To reveal a possible influence of TNF-α on lentiviral transduction susceptibility, PBMC-derived DCs were propagated in the absence of TNF-α for 8 days. After 8 days of culture, these DCs were also less transducible.

We subsequently sought to transduce blood macrophages with the lentiviral vector system. Macrophages were generated from the adherent fraction of PBMCs by culture in medium supplemented with GM-CSF for 8 days. Monocyte-derived macrophages in the cultures became firmly adherent to the tissue culture plates, as described previously (20). Those cells did not develop the typical shape and processes of DCs (Fig. 5C). In flow cytometric studies, the macrophages were distinguished from remaining lymphocytes (less than 10% after washing of nonadherent cells) by their high FSC and SSC profiles. In contrast to DCs, the macrophages on average showed lower FSC properties. The immunophenotypic analyses demonstrated that the monocyte-derived macrophages expressed CD14, but only background levels of CD1a or CD83 were detected on the cell surface.

FIG. 5. Transduction of human monocyte-derived macrophages (n = 5) by the lentiviral vector HIV-eYFPΔenvΔvifΔvpr (VSV-G pseudotyped) after 8 days in GM-CSF-supplemented culture. (A) Transduction efficiency at various m.o.i.’s (arithmetic mean with maximum/minimum). Transduction rates were assayed by determining the eYFP expression 4 to 5 days after vector exposure by FACS analysis. (B and C) Phase-contrast and fluorescence microscopy of eYFP-transduced monocyte-derived macrophages. Adherent macrophages were transduced at day 8 of culture at an m.o.i. of 100. About 25% of the cells are eYFP-positive.
transfer, which is in agreement with previous reports (6, 15). However, the transduction rates in macrophages were slightly lower in comparison to those seen in immature DCs.

To address the question if the HIV-1-based lentiviral vector is capable of integration into the cellular genome of the transduced DCs and macrophages, an Alu-LTR PCR was performed using oligonucleotide primers specific for the human Alu sequence and the HIV-1 LTR (19). HIV-1-specific DNA sequences in the Alu-LTR PCR products were demonstrated by Southern hybridization with a radiolabeled probe corresponding to a fragment of the HIV-1 LTR. Alu elements are repetitive DNA sequences disseminated throughout the eukaryotic genome. Because the HIV-1 provirus randomly integrates at multiple sites, the sizes of the Alu-LTR PCR fragments should be variable. As depicted in Fig. 6, the gel electrophoresis and Southern hybridization of the PCR products revealed a smear, which is consistent with this assumption. In macrophages and DCs, which had been transduced at culture day 3 and day 8, the Southern analyses showed hybridization of the Alu-LTR PCR products with the HIV-1-specific probe (Fig. 6, left panel, lanes B, C, and D). In contrast, no hybridization signal was observed for macrophages and DCs that had been exposed for 30 min to the lentiviruses and immediately lysed (Fig. 6, left panel, lanes A and E). Thus, the lentiviral vector integrates into the cellular genome of transduced cells.

The effect of lentiviral transduction on the DCs’ antigen-presenting function was temporarily assessed by allogeneic MLRs. Transduced and untransduced DCs were sorted according to their high FSC and high SSC profiles (Fig. 2A) and to their fluorescent characteristics (Fig. 2B), respectively. The lymphocyte stimulatory capacity of hybridized DCs was determined on day 7 of the assay by pulsing with $[^{3}H]$thymidine. The representative result of one of three experiments is shown as $[^{3}H]$thymidine incorporation (cpm (counts per minute) of the effector cells alone was below 300.

FIG. 6. Detection of integrated HIV-1 provirus in transduced macrophages and DCs. Genomic DNA of lysed cells was submitted to PCR using an Alu-specific (forward) and an LTR-specific (reverse) primer. The PCR products were separated on a 0.8% agarose gel (left panel) and subsequently analyzed by Southern blot hybridization with an LTR-specific probe (right panel). (Lane A) Macrophages were lysed 30 min after lentiviral vector exposure. (Lane B) Macrophages were lysed 4 days after transduction. (Lanes C and D) DCs were transduced at day 3 (C) and at day 8 (D) of culture and lysed 4 days later. (Lane E) DCs lysed 30 min after vector exposure. The DNA markers are 100- and 1-kb ladders (New England Biolabs).

FIG. 7. Allogeneic mixed lymphocyte reaction (MLR). DCs were transduced at day 3 of culture in GM-CSF and IL-4 and sorted according to FSC/SSC and fluorescence characteristics (Fig. 2) at day 8. Untransduced and eYFP-expressing DCs were cultured in graded doses with constant numbers of allogeneic PBLs as effectors in triplicate. Stimulatory function of DCs was determined on day 7 of the assay by pulsing with $[^{3}H]$thymidine. The representative result of one of three experiments is shown as $[^{3}H]$thymidine incorporation. The cpm (counts per minute) of the effector cells alone was below 300.
eYFP-expressing DCs was compared with untransduced DCs in graded doses as stimulatory APC. In three comparable experiments there was no obvious difference in [3H]thymidine incorporation by effector lymphocytes which had been stimulated by transduced or untransduced DCs (Fig. 7). This result suggests that lentiviral gene transfer to human PBMC-derived DCs does not impair the DC antigen-presenting function as defined by the ability to stimulate allogeneic lymphocyte proliferation.

In summary, this study demonstrates that human PBMC-derived DCs can be transduced by lentiviral vectors. Interestingly, the transduction efficiency in mature DCs was considerably lower compared to immature DCs. This result is consistent with reports that the reverse transcription of HIV-1 virus is inhibited in mature DCs, but not in immature DCs (21, 22). However, we have demonstrated efficient transduction of immature DCs with persistent transgene expression even after the cells had acquired the phenotype of mature DCs.

DCs are the most potent antigen-presenting cells for inducing differentiation of naïve CD4+ and CD8+ T-cells into helper and cytotoxic T-cells (CTL), respectively, and for initiating primary and secondary immune responses (2). DCs can be directly loaded with antigens, peptides, or tumor extracts to induce immune responses. However, genetic modification of DCs may be more effective in inducing immune responses by producing as well as processing antigens. Thus far, only retroviruses and adenoviruses have been successfully used to transduce DCs (4, 23). Adenoviruses are not ideal vectors due to their immunogenicity and the transient nature of gene expression. Although retroviral vectors have been used to efficiently transduce human CD34+ dendritic cell progenitors (24, 25), retroviral transduction of PBMC-derived DCs is technically challenging and requires multiple transduction cycles. The retroviral transduction efficiency in PBMC-derived DCs is low (23). The results of the presented study indicate that lentiviral vectors, in addition to retroviral and adenoviral vectors, may be useful to deliver a gene into PBMC-derived DCs for immunotherapy.

This study also demonstrates that macrophages can be transduced by lentiviruses, in accordance with previous reports (6, 15). Since macrophages and T-cells are major targets for HIV and play an important role in the pathogenesis of HIV, transduction of both types of cells with anti-HIV genes would be necessary for effective HIV gene therapy. Because macrophages are not susceptible to most of the gene transfer vectors, currently described HIV gene therapy approaches mainly focus on protection of T-cells. Lentiviral vectors capable of transducing both macrophages and T-cells may have significant advantage over other vectors for HIV-1 basic research and gene therapy.

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