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Comparison of Lipid-Mediated and Adenoviral Gene Transfer in Human Monocyte-Derived Macrophages and COS-7 Cells

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ABSTRACT

Lipid-mediated transfection was compared to adenoviral-mediated gene transfer in COS-7 cells as well as human monocyte-derived macrophages (HMDM). For this purpose, we monitored enhanced green fluorescent protein (EGFP) expression by fluorescence microscopy and quantified gene transfer by competitive PCR. Transfection of COS-7 cells with a novel lipid formulation for DNA transfer was highly effective in COS-7 cells. On average, 30% of the cells were fluorescent 48 h after transfection. In HMDM, the same formulation resulted in the expression of EGFP in less than 0.5% of cells. We measured plasmid DNA by quantitative PCR in lipid-transfected macrophages and found that each macrophage contained on average 2 fg of plasmid DNA 24 h after transfection, that is, more than 400 molecules of plasmid DNA entered each cell. Despite the high level of reporter DNA in lipid transfected cells, expression of the fluorescent protein was suppressed in more than 99.5% of the macrophages. We also used

adenoviral gene transfer to introduce the foreign DNA into both COS-7 cells and HMDM. Even though the multiplicity of infection was less than 30, expression of EGFP was observed in nearly all COS-7 cells and in more than 80% of HMDM 48 h after transfection. Despite major advances in the field of lipid-mediated transfection of HMDM, the lipid formulations that are available commercially cannot compete with the efficiency of adenoviral gene transfer.

INTRODUCTION

Macrophages play an important role in host immune reactions. They act as mediators of both the humoral and the cellular immune response. Because macrophages are crucial players in pathologies such as human immunodeficiency virus (HIV) infection, autoimmune diseases and chronic inflammatory diseases, they are important targets of research and clinical intervention. Both basic research and applied gene therapy approaches involving human monocytes or human monocyte-derived macrophages (HMDM) require the manipulation of gene expression of these cells.

A range of different transfection techniques for the introduction of foreign DNA into HMDM was recently developed (4,6,7,9,10,11,15). Despite the fact that several new lipid formulations for cell transfections are now commercially available (from Clontech Laboratories, Hoffmann La Roche, Qiagen and Promega, for example), lipid-mediated gene transfer to HMDM was reported by only one group, to our knowledge (4). The authors used a highly sensitive reporter gene assay to detect foreign gene expression. However, they only indicate relative levels of gene expression without calculating actual transfection efficiencies. Therefore, it cannot be excluded that the expression was limited to only a few cells. To date, efficient foreign gene expression in HMDM on a single-cell level was only observed after adenovirus-mediated gene transfer (9,11).

The genetic engineering of an adenovirus is far more complicated and demands far stricter safety considerations than the propagation of plasmids in *Escherichia coli*. This has prevented adenoviral-mediated gene transfer from be-

coming a standard molecular biological tool. A simple, less expensive but equally efficient gene delivery method would be of considerable use in many different fields of research. It was recently shown that the transfection of primary human and dog thyroid cells with a novel lipid blend, FuGENE™ (Roche Molecular Biochemicals, Basel, Switzerland) was quite effective (13). We compared FuGENE-mediated transfection to adenoviral-mediated gene transfer in COS-7 cells as well as HMDM. Expression efficiency was monitored with reporter plasmids coding for enhanced green fluorescent protein (EGFP).

MATERIALS AND METHODS

Isolation and Culture of Human Monocyte-Derived Macrophages

Human mononuclear cells were isolated from buffy coats as described (14). The buffy coats were obtained from the Red Cross blood donation center in Basel and prepared from blood samples collected from apparently healthy volunteers. Mature macrophages were plated either at a density of 0.15×10^6 cells/well on Aclar 22 mm diameter, transparent fluorinated-chlorinated thermoplastic film (ProPlastics, Linden, NJ, USA) in 12-well plates (for fluorescence microscopy), or at a density of 0.4×10^6 cells/well on 35 mm diameter Aclar in 6-well plates (for DNA-isolations).

The cell culture medium consisted of RPMI supplemented with 2 mmol/L N-acetyl-L-alanyl-L-glutamine, 1% vol/vol nonessential amino acids, 100 U/L and 100 µg/mL, respectively, of penicillin G/streptomycin (all from Biochrom, Allschwil, Germany) and 10 mM HEPES (Fluka Chemie, Buchs, Switzerland).

Endotoxin levels of the culture medium and all buffers were tested routinely and were below 0.03 enzyme U/mL. Cells were seeded in culture medium supplemented with 2% autologous human serum. After 2 h, cells were transferred into culture medium containing 5% autologous human serum and incubated for 48 h before transfections or adenoviral infections. This cell isolation technique did not select for any mono-

cyte subpopulations, and macrophage adherence was quantitative (14).

COS-7 Cells

COS-7 cells (ATCC No. CRL-1651) were cultured in IMDM with 25 mmol/L HEPES (Bio Whittaker, Belgium), supplemented with 2 mM N-acetyl-L-alanyl-L-glutamine, 100 U/L and 100 µg/mL, respectively, of penicillin G/streptomycin (both from Biochrom) and 10% heat-inactivated fetal bovine serum (FBS; Flow Laboratories, McLean, VA, USA). Cells were not used after passage 30. One day before transfection or infection, cells were seeded at 40% confluency into either 12-well plates on Aclar for fluorescence microscopy or directly into 6-well plates for DNA extraction. On the day of transfection, cell density was routinely 5×10^4 cells per cm².

Plasmids

The plasmid pCMX-PL2 (12), containing the open reading frame (ORF) of EGFP (a gift from Dr. Hashemolhosseini, Baxter, Germany), was cut within its cloning site with *EcoRV* and *HindIII*. The remaining vector was Klenow filled-in and blunt-end ligated, which resulted in a plasmid (pCMX-PL2-EGFPdel) 31 bp shorter than the parent vector. The parent vector was used as the competitor in quantitative PCR. The two plasmids were propagated in *E. coli* DH5α. The pCMX-PL2-EGFPdel was purified with the EndoFree Kit (Qiagen, Basel, Switzerland), and the plasmid pCMX-PL2-EGFP with the plasmid purification kit (Macherey-Nagel, Oensingen, Switzerland).

Recombinant Adenovirus Generation

Virusmid vmAd-CG, a cloned full-length recombinant adenoviral genome expressing EGFP under control of the CMV promoter, was generated in bacteria according to published methods (3). This was carried out by homologous recombination of *SwaI*-linearized virusmid vmRL-CMV1 with a 5217 bp *Pfl-MI-FspI* fragment from plasmid pSTC-EGFP/BAD-kz encompassing a CMV-EGFP-β-globin IVS2/pA expression

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unit. The VmRL-CMV1 contains a full-length $\Delta E1\Delta E3$ adenovirus 5 backbone flanked by *PacI* restriction sites. Its deleted E1 region harbors a partial CMV-promoter collapsed with a rabbit β globin IVS2/pA unit to generate a unique invasive *SmaI* site. This configuration allows for the direct recombinational insertion of any ORF from vectors bearing the corresponding expression cassette elements, as is the case for plasmid pSTC-EGF/BAD-kz. Plasmid sequences are available on request.

Recombinant adenoviruses were generated by transfection of 4 μ g of *PacI*-digested vmAd-CG into HER911 cells and propagated in the same cell line. Virus manipulations were done according to standard protocols (8), and viral titers were determined as TCID₅₀ infecting HER911 cells with variable dilutions of virus stock. Calculations were performed according to the method of Reed and Munch (2).

Transfections and Adenoviral Infections

Lipid-mediated transfections with FuGENE were carried out according to the protocol supplied by the manufacturer with a DNA to FuGENE ratio of 1:3 (wt/vol). Briefly, 1 μ g of pCMX-PL2-EGFPdel was covered with a mixture containing 3 μ L of FuGENE and 50 μ L of medium without serum. This transfection cocktail was carefully added to 4×10^5 HMDM or 5×10^5 COS-7 cells, respectively, in 1.5 mL of culture medium per well of a 6-well plate.

Replacing the culture medium and serum withdrawal had no effect on the transfection efficiency. The same amount of plasmid DNA (1 μ g) without the lipid formulation was added to cells as a control. Cells were then incubated with 50 μ g DNaseI per mL of medium to remove any DNA nonspecifically adsorbed to the cell surface. For adenoviral infections, cells growing on Aclar were transferred to low-serum culture medium. For COS-7 cells and HMDM,

this medium consisted of the respective culture medium supplemented with either 2% FBS for COS-7 cells or 2% human serum for HMDM. These media contained adenovirus at 30 MOI. After 2 h, the low-serum culture media were exchanged for media containing no adenovirus and the standard percentages of serum, 10% FBS for COS-7 cells and 5% human serum for HMDM, respectively.

Quantitative PCR

Cells were lysed for 1 h at 50°C in 50 mmol/L Tris-HCl buffer (pH 8.0) containing 10 mmol/L EDTA, 0.5% SDS and 0.5 mg Proteinase K/mL. Total DNA in the lysate was isolated by two rounds of extraction with phenol/chloroform/isoamylalcohol (25:24:1 vol/vol/vol, saturated with 100 mmol/L Tris-HCl, pH 8.0) and subsequent ethanol precipitation. The T7 primer (5'-TAATACGACTCACTATAGGG-3'), annealing on pCMX-PL2-EGFP at position -54 to -35 relative to the start

Figure 1. COS-7 cells transfected with plasmid pCMX-PL2-EGFPdel. Cells were transfected with EGFP by lipid-mediated gene transfer using the lipid blend FuGENE. Cells were fixed and mounted either after 24 h (panels A and B) or 48 h (panels C and D). Panels A and C are Hoffmann-contrast images of panels B and D, respectively. Bar is 40 μ m.

codon, was used as the sense primer, and the antisense primer (5'-GCAGAT-GAACTTCAGGG-3') stretched from position 129 to 145 within the ORF of EGFP. The two primers gave rise to a 201 bp PCR product on pCMX-PL2-EGFPdel, and the same primers resulted in a 232 bp product on the parent plasmid pCMX-PL2-EGFP. For PCR, 38 μ L of a PCR mixture containing (final concentrations) 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8 at 25°C), 0.08% Nonidet P-40, 1.5 mmol/L MgCl₂, 0.1 mmol/L dNTPs, 0.2 mmol/L primer (each) and 0.5 U *Taq* DNA polymerase (MBI-Fermentas, Basel, Switzerland) were added to 2 μ L of template DNA. Template DNA consisted of either 1 μ L of cell-derived DNA plus 1 μ L of water or 1 μ L of cell DNA, to which 1 μ L of the competitor plasmid pCMX-PL2-EGFP was added at the indicated concentrations. Cycling was performed on a Hybaid PCR Sprint Thermocycler with a profile consisting of an initial denaturation step of 2 min at 94°C, followed by 30 cycles of 30 s each at 94°C, 48°C and 72°C, respectively, and a final extension step of 5 min at 72°C.

Fluorescence Microscopy

Cells were harvested either 24 h or 48 h after transfection or adenoviral infection. Cells were washed twice with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Fixed cells were washed twice in PBS and mounted in the embedding medium Mowiol containing 1% n-propyl gallate. Cells were examined using a Diaphot 300 fluorescence microscope (Nikon).

RESULTS AND DISCUSSION

Lipid-Mediated Transfection

Recently, a variety of new lipid formulations for the transfection of mammalian cells became commercially available. These systems rely on the interaction of a cationic lipid molecule with plasmid DNA. The DNA-lipid complex is taken up by the cells. After being released from the endosomes, the plasmid DNA enters the nucleus by a yet unknown mechanism. In our studies, we

tested the non-liposomal lipid blend FuGENE for the transfection of COS-7 cells as well as HMDM. This lipid formulation was designed for the highly effective transfection of primary cell types.

The COS-7 cells, transfected by the lipid-mediated technique with pCMX-PL2-EGFPdel, effectively express the GFP at both 24 h and 48 h after transfection (Figure 1). Typical green fluorescence was observed on average in 30% of the cells. With increasing passage numbers, the percentage of green fluorescent cells decreased (not shown). This transfection method did not require additional changes of medium. Also, the relatively high serum content (10%) did not interfere with the transfection efficiency. In contrast, expression of pCMX-PL2-EGFPdel in HMDM subjected to this lipid-mediated transfection technique routinely yielded low transfection rates, with less than 0.5% cells expressing EGFP (not shown). We also tested three other commercially available lipid formulations; however, none of these systems was any more effective than FuGENE in transfecting HMDM (not shown).

We therefore analyzed whether the plasmid DNA had actually entered the cells after treatment with FuGENE, and so we performed competitive PCR. The plasmid alone did not penetrate the cells, and no amplified DNA was detected by PCR either in COS-7 cells or in HMDM, even without competitor DNA being included in the PCR (Figure 2; no DNA and DNA only). In contrast, lipid-associated plasmid DNA effectively entered the cells and was detectable in COS-7 cells and HMDM by competitive PCR, even in the presence of 150 pg and 15 pg, respectively, of competitor DNA (Figure 2; transfected). For PCR, we used 1/60 of the DNA isolated from one well. Hence, for COS-7 cells, this translates to at least 9000 pg pCMX-PL2-EGFPdel per 5×10^5 cells, or 18 fg corresponding to 3600 plasmid molecules per cell after the 24 h transfection period, assuming a molecular mass of the plasmid of 3×10^6 .

The amount of plasmid increased further during the subsequent 24 h incubation periods (Figure 2, COS-7, 48 h). The COS-7 cells are simian virus 40 (SV40) transformed cells and therefore can propagate the SV40 origin of repli-

cation-containing plasmid pCMX-PL2-EGFPdel. This is the likely reason for the relatively high amount of plasmid DNA within those cells. To test the contribution of the SV40 origin of replication, we transfected HeLa cells, which are not SV40 transformed, exactly as described above. The percentage of fluorescent HeLa cells was only slightly lower than the one for COS-7 cells (5%–10%), even though the amount of DNA penetrating into the cells equaled only 60 plasmid molecules per HeLa cell. We also observed an increase in the amount of plasmid molecules from 24 to 48 h (data not shown). This increase is most likely due to continued intracellular accumulation of DNA rather than plasmid propagation.

For HMDM, we obtained yields of more than 2 fg plasmid per cell corresponding to 400 plasmid molecules 24 h after the initiation of transfection. As

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in COS-7 cells, the number of plasmid molecules increased during the following 24 h. However, similar to HeLa cells, the plasmid cannot be propagated in HMDM, which suggests that plasmid molecules continue to accumulate in HMDM even after 48 h. Despite the high intracellular concentration of plasmids, more than 99.5% of macrophages did not express the foreign gene at a detectable level. This result suggests that the endogenous plasmid DNA was either trapped in an endosomal compartment within the macrophages or intranuclear delivery was severely hampered. Studies by Mortimer et al. (16) favor the latter possibility.

Adenoviral-Mediated Gene Transfer

After they have entered the cell, adenoviruses (1) and adenoviral particles (5) can disrupt the endosomal compartment, which releases the associated DNA into the cytosol where the DNA can migrate into the nucleus. When we used adenoviral vectors for gene delivery at an MOI of 30, EGFP expression efficiency increased dramatically compared to lipid-mediated gene transfer, as shown in Figure 3. Essentially, all COS-7 cells expressed the foreign gene. Even if all viruses had penetrated into cells, no more than 1% of the amount of plasmid DNA found in FuGENE-transfected cells would be present in adenovirus-infected COS-7 cells. Adenoviral-infected COS-7 cells, after longer incubation periods, tended to overgrow. The infection obviously eliminated their natural contact inhibition (Figure 3, A and B; 48 h).

Based on the MOI of 30, adenovirus-infected HMDM contain at best 10% of the amount of plasmid DNA found in FuGENE-transfected HMDM. Nevertheless, more than 80% of the cells reproducibly expressed EGFP 48 h after infection (Figure 3D; 48 h). HMDM were fully viable and continued to express the foreign gene even after 7 days of incubation (data not shown). Therefore, unlike COS-7 cells, HMDM do not appear to be affected by adenoviral infection. Because of the strong fluorescence signal emitted by infected HMDM, adenoviral-mediated EGFP delivery to HMDM might prove useful for the study of such processes as apop-

Figure 2. Competitive PCR on COS-7 and HMDM-derived plasmid DNA. COS-7 cells and HMDM were either transfected with the FuGENE-complexed plasmid pCMX-PL2-EGFPdel (transfected), or incubated in the presence (plasmid only) or absence of the plasmid (no plasmid). Total DNA was isolated from the cells, and PCR was performed either in absence of competitor (no plasmid and plasmid only) or in the presence of the indicated amounts of competitor DNA (plasmid pCMX-PL2-EGFP).

Figure 3. Adenovirus-mediated EGFP expression in COS-7 cells and HMDM. COS-7 cells (panels A and B) and HMDM (panels C and D) were grown on Aclar in 12-well plates in normal cell culture media. Before adenoviral infection, supernatants were replaced with media supplemented with either 2% heat-inactivated fetal calf serum (COS-7 cells) or 2% heat-inactivated human serum (HMDM), respectively. Adenovirus (30 MOI) carrying the ORF of EGFP were added to these culture media. After 2 h, supernatants were replaced with fresh culture media with 10% serum (COS-7 cells) and 5% serum (HMDM), and cells were incubated for the times indicated. Subsequently, cells were fixed and mounted. Panels A and C represent Hoffmann-contrast images of panels B and D, respectively. Bar is 40 μ m.

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tosis, cell migration or other studies requiring the tracking of individual cells.

In conclusion, the novel lipid blend FuGENE is a useful tool for the transfection of cell lines such as COS-7 cells. This and other commercially available lipid blends are inexpensive, easy to use and yield high transfection rates. Transfection of HMDM with FuGENE might prove useful in single-cell studies where, due to the genetic manipulation, obvious morphological changes are detectable. However, only adenoviral-mediated gene transfer strategies result in the expression of genes of interest in a sufficiently high number of target cells (>80%) to study macrophage function on a biochemical level.

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