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mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers

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ABSTRACT

Messenger RNA encoding luciferase (mLUC) was complexed to the cationic lipids Lipofectamine or DOTAP/DOPE, and to the cationic polymer linear poly(ethyleneimine) (linPEI). The complexes were incubated with HeLa cells and luciferase expression was assessed. The type of non-viral carrier used determined the extent and duration of protein expression. Maximal duration of mRNA expression was about 9 days for Lipofectamine complexes, i.e. not very much shorter than with pDNA polyplexes. Interestingly, luciferase activity was already detected 30 min after adding the mRNA complexes to the cells, independent on the type of carrier. We also assessed the proportion of cells that become transfected by means of transfection with an mRNA encoding GFP. For both cationic lipids transfection with mRNA yielded a substantially larger fraction of transfected cells (more than 80%) than transfection with pDNA (40%). In addition we tested the carriers for their ability to mediate delivery of mRNA encoding CXCR4 into mesenchymal stem cells. The fraction of CXCR4-positive cells obtained with the mRNA–cationic lipid complexes was around 80%, as compared to 40% for the linPEI polyplexes. Our results demonstrate that the advantage of the use of mRNA over that of pDNA may under certain conditions outweigh the disadvantage of the somewhat shorter expression period.

1. Introduction

It is well known that cationic carriers can condense plasmid DNA into positively charged complexes. Such complexes can interact with the plasma membrane and be taken up by cells, most commonly by endocytosis [1–3]. To ensure transfection, the complexes have to escape the endosomal compartment and release pDNA into the cytosol. In the last step the DNA needs to enter the nucleus. It has been shown that the nuclear envelope represents a serious obstacle for the entry of pDNA into the nucleus, especially in non-dividing cells [4,5]. Therefore, in an attempt to bypass the nuclear envelope we investigated the possibility of transfecting cells with mRNA instead of pDNA.

Besides the fact that nuclear delivery is not required for mRNA to be effective, mRNA-mediated transfection may hold several other advantages as well. Most importantly, there is no danger of introducing irreversible genomic modification. Moreover, in contrast to plasmid DNA, messenger RNA is devoid of immunogenic CpG motifs.

Since the first study reported by Malone and colleagues [6] surprisingly little attention has been paid to the use of messenger RNA (mRNA) for the transfection of cells, which may be explained by the wide-held belief that mRNA is too unstable to ever be used as a drug. So far, only few studies focused on complexation of mRNA with cationic carriers (like cationic lipids and cationic polymers) in the last decade. As a consequence knowledge concerning mRNA-mediated transfection is still very scarce [7–12]. In addition to the use of cationic lipids and polymers also electroporation turned out to be a useful method for mRNA delivery to human embryonic and hematopoietic cells *in vitro* [13–15].

Successful mRNA-based applications will require sufficient knowledge on kinetics of the production of the protein of interest. The desired time frame of protein production will depend on the cell type to be transfected and the specific application. It is to be expected that both the onset and duration of protein expression will strongly depend on the nature of the mRNA carrier used as well as on the *way* the carrier/mRNA complexes are prepared. It is precisely for this reason that we set out to perform the research described in this paper: characterization of the transfection potential of mRNA complexes formed from mRNA and cationic lipids and polymers (Lipofectamine, DOTAP/DOPE, linear PEI), including a direct comparison with pDNA.

2. Materials and methods

2.1. Cell culture

HeLa cells were cultured in Dulbecco's Modified Eagle's culture medium containing the growth factor F12 and phenol red (DMEM: F12) and supplemented with 2 mM glutamine, 10% heat-inactivated FBS and 100 U/ml penicillin/streptomycin. mMSC (a kind gift from

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Prof. Catherine Verfaillie, Stem Cell Institute, KULeuven, Belgium) were cultured in Iscove's Modified Dulbecco's Medium (IMDM — BioWhitteker) supplemented with 2 mM glutamine, 10% heat-inactivated FBS, 10% horse serum and 100 U/ml penicillin/streptomycin. The cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. All cell culture products were purchased from Gibco/Invitrogen (Merelbeke, Belgium). Cells were seeded on 24-well plates 24 h before transfection.

2.2. Nucleic acids

2.2.1. Plasmid DNA

A 4.7 kb pDNA encoding green fluorescent protein (pEGFP-N1) was purchased from Clontech, (Leusden, The Netherlands) and pGL4.13 (4641 bp) encoding firefly luciferase was bought from Promega Corporation (Leiden, The Netherlands). pGEM4Z-EGFP-A64 (kindly provided by Prof. Dr. E. Gilboa, Duke University Medical Center, Durham, NC, USA) was previously described by Nair et al. [16]. pBlue-Luc-A50 was earlier described by Sheets et al. [17]. Plasmid encoding CXCR4 was a kindly provided by Dr. Peter Ponsaert (Laboratory of Experimental Hematology, University of Antwerp, Belgium).

2.2.2. Messenger RNA

To produce mRNA by *in vitro* transcription, appropriate plasmids (pGEM4Z/EGFP/A64 or pBlue-Luc-A50 or pCXCR4) were first purified using a QIAquick PCR purification kit (Qiagen) and linearized using restriction enzymes (Dra I for plasmid encoding firefly luciferase or Spe I for plasmid encoding GFP or Xba I for plasmid encoding CXCR4, respectively). Linearized plasmids were used as templates for the *in vitro* transcription reaction using the T7 mMessage mMachine kit according to the manufacturer's instructions. This resulted in production of mLuc and mGFP that were both capped and polyadenylated. mCXCR4 was polyadenylated with a poly(A) tailing kit provided by Ambion. mRNAs were purified by DNase I digestion followed by LiCl precipitation and 70% ethanol wash. The mRNA concentration was determined by measuring the absorbance at 260 nm. mRNA was stored in small aliquots at −80 °C at a concentration of 1 μg/μl.

2.3. Preparation of nucleic acid complexes

2.3.1. mRNA polyplexes

Linear Polyethyleneimine (jetPEI™) was purchased from PolyPlus Transfection (Illkirch, France). The concentration of the stock solution was 7.5 mM. 2, 3, 4, 5 or 6 μl of linPEI were dispersed in 48, 47, 46, 45 or 44 μl of sodium chloride solution (150 mM) (PolyPlus Transfection, Illkirch, France). This 50 μl solution was then added to 50 μl of mRNA solution, containing 2 μg mRNA (1 μg/μl) in 48 μl of sodium chloride solution. After 10 min of incubation at room temperature, 900 μl of OptiMem was added (Invitrogen, Merelbeke, Belgium). Aliquots of the complete 1 ml solution were added to the cells.

2.3.2. mRNA lipoplexes

A mixture of DOTAP and DOPE (molar ratio 1:1; in chloroform) was purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). To make DOTAP/DOPE liposomes, 100 μl of a DOTAP/DOPE chloroform solution (10 mg/ml) was transferred to a sterile glass flask. A lipid film was formed on the glass surface by evaporating the solvent under nitrogen atmosphere. The addition of 1 ml of nuclease-free water in the presence of sterile glass beads was followed by sonication, yielding DOTAP/DOPE liposomes. The total lipid concentration in these liposome dispersions was 1 mg/ml. 2, 4, 6, 8, 10 or 12 μl of this liposome dispersion was dissolved in 48, 46, 44, 42, 40 or 38 μl of OptiMem. This 50 μl solution was then added to 50 μl of mRNA solution, containing 2 μg mRNA (1 μg/μl) in 48 μl OptiMem. After 10 min of incubation at room temperature, 900 μl of OptiMem was added. Aliquots of the resulting 1 ml solution were added to the cells.

Lipofectamine™ 2000 was purchased from Invitrogen (Merelbeke, Belgium). The concentration of the stock solution was 1 mg/ml. 2, 4, 6, 8 or 10 μl of this solution was dissolved in 48, 46, 44, 42 or 40 μl of OptiMem. This 50 μl solution was then added to 50 μl of mRNA solution, containing 2 μg mRNA (1 μg/μl) in 48 μl of OptiMem. After 10 min of incubation at room temperature, 900 μl of OptiMem was added. Aliquots of the resulting 1 ml solution were added to the cells.

2.3.3. pDNA complexes

The protocol employed to prepare pDNA complexes was the same as described above for mRNA complexes. The ratio of cationic carrier/pDNA was optimized based on the manufacturer's instructions. Briefly, 4 μl of linPEI or 20 μl of DOTAP/DOPE or 4.6 μl of Lipofectamine were complexed with 2 μg of pDNA.

2.4. Transfection of cells

Experiments were performed in 24-well plates (30 000 cells/well). If not specified otherwise, cells were incubated with the complexes for 4 h. Incubation times between 2 and 4 h led to similar transfection efficiencies. Subsequently, the complexes were removed and fresh culture medium was added. Luciferase activity was assayed after different periods of time. If luciferase activity was detectable for more than 2 days, the cell cultures were split every two days. One half was analysed for luciferase expression and the other half was further cultured. GFP and CXCR4 production was assayed in 12-well plates (60 000 cells/well) 24 h after transfection.

2.5. Bioluminescence assay

After removing the culture medium, the cells were washed twice with PBS (Gibco/Invitrogen, Merelbeke, Belgium). Subsequently, 100 μl of Cell Culture Lysis Reagent (Promega, Leiden, The Netherlands) was added to the cells for 30 min. The samples were then centrifuged (12 000 rpm at 4 °C for 5 min) and 40 μl supernatant of the samples was transferred to a 96-well plate. Luciferase activity of each sample was assayed in a GloMax™ 96 Luminometer which added 100 μl of the substrate solution in each well and measured the light emitted over a 10-s period. The protein content of each sample was determined by a standard Bradford assay (Biorad, Nazareth Eke, Belgium). The results are expressed as relative light units (RLU) per milligram of protein.

2.6. Flow cytometry

To assess numbers of GFP-positive cells, culture medium was removed from the wells and the cells were washed with PBS. After detaching the cells with trypsin (0.25%, Gibco/Invitrogen, Merelbeke, Belgium) and centrifugation, the cells were re-suspended in flow buffer (PBS containing 1% BSA and 0.1% azide). The samples were kept on ice until GFP expression was evaluated with a Beckman Coulter flow cytometer FC500, equipped with a 488-nm laser. To evaluate numbers of CXCR4 positive cells, culture medium was removed from the wells and the cells were washed with PBS. After detaching the cells with acutase (Sigma-Aldrich, Belgium) and centrifugation, the cells were re-suspended in PBS. After washing, 5 μl of a fluorescently labelled antibody was added (Phycoerythrin Rat Anti-Mouse CD184 (CXCR4) BD Bioscience Pharmingen, Belgium) and incubated with the cells for 15 min at 4 °C. After a washing in PBS, the cells were re-suspended in flow buffer (PBS containing 1% BSA and 0.1% azide). The samples were kept on ice until CXCR4 expression was evaluated by a flow cytometer (BD FACSCalibur 4) equipped with a 635-nm laser.