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mRNA but not plasmid DNA is efficiently transfected in murine J774A.1 macrophages

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Abstract

Previous studies demonstrated that macrophages are difficult to transfect. In the present study, we investigated whether J774A.1 macrophages can be efficiently transfected using nucleofector technology. Nucleofection of J774A.1 macrophages with mRNA resulted in transfection efficiencies up to 75% without cell death as compared to control pulsed macrophages. In contrast, introduction of DNA into J774A.1 cells caused apoptosis without expression of the gene of interest. Our results show that mRNA nucleofection is a new high-speed transfection method for macrophages.
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Keywords: Gene transfer; Green fluorescent protein; J774A.1 macrophage; Nucleofection; Apoptosis

Studies on the regulation of gene expression in macrophages have only occasionally been reported most likely because these cells are difficult to transfect. Indeed, the transfection efficiency of murine J774A.1 macrophages using gene transfer methodologies such as electroporation, lipofection, diethylaminoethyl dextran or particle bombardment is below 20% [1]. Recently, we used nucleofector technology to transfect the hard-to-transfect human monocytic cell lines U937 and THP-1, and we obtained transfection efficiencies up to 80% without significant cell toxicity [2]. Nucleofector technology is a non-viral, electroporation-based transfection method that transports DNA directly into the cell nucleus and that consists of two different components: (i) a nucleofector device which delivers unique electrical parameters and (ii) cell-type specific solutions in which the cells are retained when the electrical program is activated. The technology is suitable for the delivery of different substrates such as DNA, RNA or short interfering RNA [3–5]. The aim of the present study was to investigate whether J774A.1 macrophages can be efficiently transfected by using this technology.

Materials and methods

Cell culture. Murine J774A.1 macrophages (American Type Culture Collection) were grown in RPMI 1640 medium (Invitrogen, San Diego, CA), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), gentamicin (50 μg ml^{-1}), and polymyxin B (20 U ml^{-1}).

Plasmids. For plasmid transfections, pEGFP-N3 DNA (Clontech Laboratories, Palo Alto, CA) was used encoding enhanced green fluorescent protein (eGFP) under control of a cytomegalovirus immediate early promoter. A pN3 plasmid without eGFP cassette was constructed by digesting pEGFP-N3 with *Not*I–*Bam*HI, blunting of ends with Klenow fragment, and self-ligation. The plasmids were propagated in *Escherichia coli* strain TOP10 F' (Invitrogen) and purified. Endotoxin-free plasmid DNA was prepared using an Endo-Free Plasmid kit (Qiagen, Chatsworth, CA). Double-stranded oligonucleotides were produced by mixing equal amounts of sense and antisense oligonucleotides from a 24 bp region of the GFP gene

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(5′CGGCAAGCTGACCCTGAAGTTCAT3′ and complementary strand). dNTP Mix, NTP Mix, and an oligo(dT)$_{20}$ were from Invitrogen.

Production of eGFP mRNA and dsRNA. Plasmid pGEM4Z-GFP-A64 (gift from Van Tendeloo, Belgium) containing the eGFP coding sequence downstream of a T7 promoter was used. A poly(A) tail of 64 residues was cloned downstream of eGFP followed by a *Spe*I restriction site. To produce eGFP mRNA, purified pGEM4Z-GFP-A64 DNA was *Spe*I linearised, purified, and used as DNA template for in vitro transcription using the T7 mMessage mMachine transcription kit (Ambion, Austin, TX). For the synthesis of GFP double-stranded RNA (dsRNA), the eGFP cassette was isolated from pGEM4Z-GFP-A64 as a *Bam*HI fragment and inserted in a *Bam*HI linearised Litmus 28i vector (New England Biolabs, Beverly, MA). The resulting plasmid Litmus 28i-GFP was linearised either by *Stu*I or *Bgl*II, purified, and used as DNA template for in vitro transcription using the T7 mMessage mMachine transcription kit (Ambion). Unincorporated nucleotides were removed by size exclusion chromatography. RNA integrity was assessed by capillary electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). RNA was stored at −80 °C in small aliquots.

Transfection of eGFP DNA, dsRNA or mRNA. J774A.1 macrophages were washed and trypsinized. After trypsin inactivation with 20% (v/v) FCS, 10^6 cells were washed and resuspended in 100 μl Cell Line Nucleofector Solution V (Amaxa GmbH, Köln, Germany). Cells were nucleofected with 1 μg pEGFP-N3, 1 μg eGFP dsRNA or 1–5 μg eGFP-specific mRNA using program T-20 of the nucleofector device (Amaxa GmbH). Thereafter, cells were immediately mixed with 500 μl prewarmed RPMI 1640 medium and transferred into well plates containing 1 ml RPMI 1640 medium per well. Cells were incubated at 37 °C for 18 h.

Evaluation of eGFP expression by flow cytometry and Western blotting. eGFP expression in nucleofected macrophages was assessed by flow cytometry (FACSort analytical flow cytometer, BD Biosciences) 1–18 h after transfection. Propidium iodide (PI, 0.8 μg ml^{-1}) was added immediately before flow cytometry to assess cell viability.

For Western blotting, transfected cells were lysed in Laemmli sample buffer (Bio-Rad, Richmond, CA). Cell lysates were heat denatured and loaded on 12.5% (v/v) SDS–polyacrylamide gels. After electrophoresis, blotting to Hybond enhanced chemiluminescence membranes (Amersham Biosciences, Freiburg, Germany) was performed according to standard procedures. Membranes were blocked in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T) and 5% (w/v) non-fat dried milk (Bio-Rad) for 1 h and were probed overnight at 4 °C with a rabbit polyclonal anti-GFP Living Colors A.v. peptide antibody (Clontech Laboratories, 1:400). Membranes were washed with TBS-T and incubated with swine anti-rabbit peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark, 1:3000) for 1 h at room temperature. After excessive washing, antibody detection was accomplished with SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL). Signals were visualised using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

Annexin V-FITC apoptosis detection. Apoptotic cell death was detected by flow cytometry using an Annexin V-Propidium Iodide Kit (BD Biosciences). Data were analysed using Cell Quest Pro software (BD Biosciences). Annexin V-positive but propidium iodide-negative cells were considered apoptotic.

Cleaved caspase-3 immunostaining. J774A.1 macrophages (10^6) transfected with 1 μg pEGFP-N3 DNA or dsRNA were grown in Falcon culture slides (BD Biosciences). Eight hours after nucleofection, cells were fixed in 4% paraformaldehyde, washed, and air-dried for 1 h. After pre-treatment with 0.2% Triton X-100 and 0.3% H_2O_2, cells were incubated with a rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signaling technology, Beverly, MA, 1:200) for 1 h. Subsequently, the slides were incubated with a goat anti-rabbit biotin antibody (Vector Laboratories, Burlingame, CA, 1:200) for 30 min and a peroxidase labelled streptavidin–biotin complex (Biogenex, San Ramon, CA, 1:100) for 45 min. For demonstration of the complex, 3-amino-9-ethylcarbazole was used as a chromogen.

Caspase-3 and PARP-1 Western blotting. Transfected cells were lysed in Laemmli sample buffer (Bio-Rad). Cell lysates were heat denatured, loaded on 7.5% or 12.5% (v/v) SDS–polyacrylamide gels, and blotted to Immobilon-P membranes (Millipore, Bedford, MA) according to standard procedures. The following antibodies were used: monoclonal anti-caspase-3 antibody (BD Biosciences, 1:1000) for caspase-3 and cleaved caspase-3, monoclonal mouse anti-poly(ADP-ribose) polymerase (PARP) antibody (BD Biosciences, 1:2000) for PARP-1 and cleaved PARP-1, monoclonal anti-β-actin (clone AC-15, Sigma, 1:5000), and rabbit anti-mouse peroxidase-conjugated secondary antibody (DAKO, 1:2000).

Neutral red viability assay. Macrophages, untreated or treated with DNA, mRNA, dsRNA or free nucleotides, were incubated with neutral red dye (10 μg ml^{-1}) in RPMI 1640 medium for 2 h. Thereafter, the dye was removed and the cells were washed twice with PBS. The incorporated dye was extracted from the cells with acidified ethanol and was measured spectrophotometrically at 540 nm. Cell viability was expressed as percent of the untreated control and was statistically evaluated in the different treatment conditions with one-way analysis of variance (ANOVA) followed by the Dunnett test. A value of $p < 0.05$ was considered significant.

DNA fragmentation. Macrophages were lysed in hypotonic lysis buffer (10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100) containing 0.5 mg ml^{-1} proteinase K (Invitrogen). Lysates were incubated for 1 h at 50 °C, then supplemented with DNase-free RNase A (5 μg ml^{-1}), and incubated for an additional hour at 37 °C. The samples were precipitated overnight (−20 °C) with isopropanol. Pellets were dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). Electrophoresis was performed on E-Gel 2% agarose (Invitrogen) for 30 min at 70 V. DNA laddering was visualised under UV light.

Results

DNA nucleofection

Nucleofection of J774A.1 macrophages with pEGFP-N3 plasmid DNA containing the eGFP coding sequence did not result in expression of eGFP protein (Fig. 1). Instead, macrophages incorporated PI (Fig. 1) but not the vital stain neutral red (Fig. 2), suggesting initiation of cell death. To investigate whether cell death was due to DNA entry and not a result of LPS-contaminated DNA or toxic expression of eGFP, J774A.1 macrophages were nucleofected with endotoxin-free plasmid DNA, with or without an eGFP cassette. All these conditions resulted in significant cell death (Fig. 2). In contrast, addition of plasmid DNA without pulsing the cells or nucleofection with either double-stranded oligonucleotides (24 nt DNA), oligo(dT)$_{20}$, dNTPs or NTPs did not result in cell death (Fig. 2).

To evaluate whether J774A.1 macrophages died via apoptosis after DNA entry, cells were incubated with annexin V-FITC (Fig. 1). Nucleofection with plasmid DNA resulted in extensive annexin V labelling as compared to cells pulsed without DNA. Despite a clear decrease in procaspase-3 after nucleofection (Fig. 3E), cleavage of caspase-3, as shown by immunocytochemistry (Fig. 3C) and Western blotting (Fig. 3E, p17 frag-

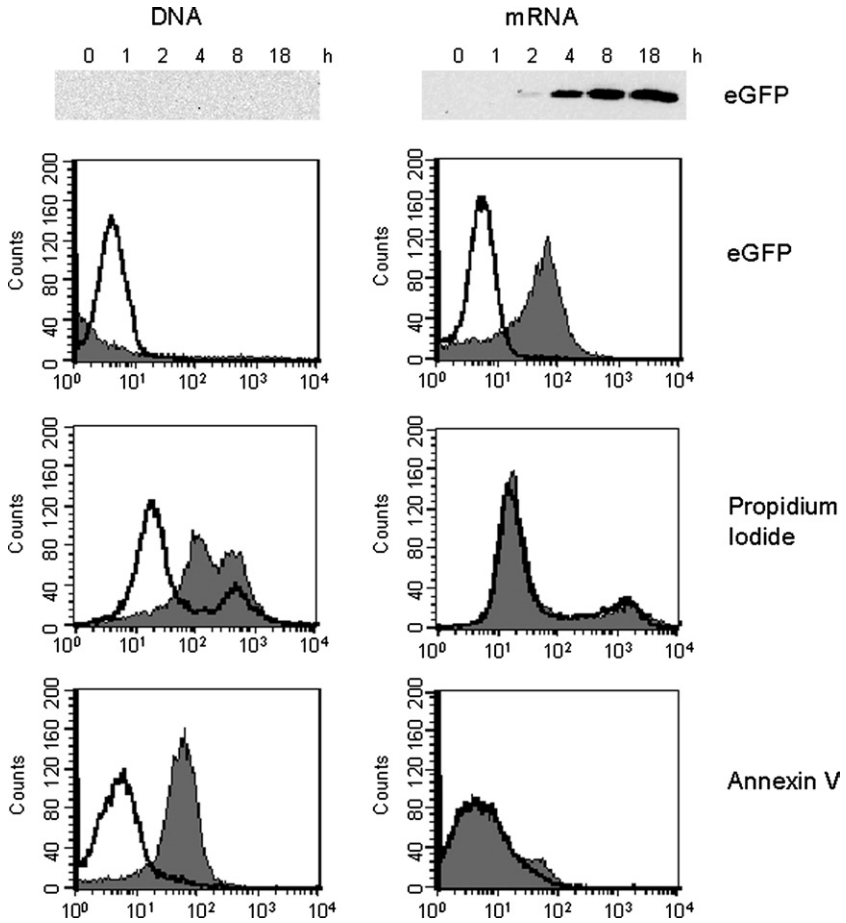

Fig. 1. eGFP expression in J774A.1 macrophages after nucleofection with 1 μg eGFP-encoding pEGFP-N3 DNA or 2 μg eGFP-specific mRNA. Kinetics of eGFP expression were determined by Western blotting. Eighteen hours after nucleofection, transfection efficiency, cell viability (propidium iodide), and phosphatidylserine externalisation (annexin V detection) of transfected cells (shaded histogram) versus controls (open histogram) were evaluated with flow cytometry. Data are representative of at least four experiments.

ment), was limited. Accordingly, PARP-1, a caspase-3 substrate, was scarcely cleaved into 85 (Fig. 3E, cleaved PARP) and 25 kDa fragments (not shown). In order to further characterise DNA induced cell death, J774A.1 macrophages were nucleofected with eGFP dsRNA, which is a known inducer of apoptosis [6]. As shown in Fig. 2, this procedure resulted in extensive cell death. After 8 h (not shown) and more pronounced after 18 h, the dsRNA transfected macrophages were clearly positive for cleaved caspase-3 (Figs. 3D and E) and cleaved PARP (Fig. 3E), indicating that dsRNA induced apoptosis. Finally, we showed that nucleofection of J774A.1 macrophages with DNA or dsRNA was associated with partial DNA fragmentation (Fig. 3F).

mRNA nucleofection

Flow cytometric analysis of eGFP expression after mRNA transfer (2 μg/10^6 cells) in J774A.1 macrophages revealed expression efficiencies ranging between 60 and 75% (Fig. 1). Increasing eGFP mRNA above 2 μg/10^6 cells did not further improve the transfer efficiency (data not shown). Both PI incorporation (Fig. 1) and neutral red viability assays (Fig. 2) demonstrated that transfer of eGFP mRNA did not result in cell death (absence of annexin V labelling and PI incorporation) as compared to controls (macrophages pulsed in absence of mRNA). Kinetics experiments showed that eGFP expression started 2 h after nucleofection (Fig. 1).

Discussion

In this study, J774A.1 macrophages were successfully nucleofected with eGFP mRNA using nucleofector technology (transfection efficiencies between 60% and 75%) without inducing significant cell death. However, transfection of J774A.1 macrophages with DNA or dsRNA was associated with extensive cell death and did not result in eGFP expression. To characterise the type of cell death, multiple techniques including flow cytometry, protein analysis, and evaluation of DNA

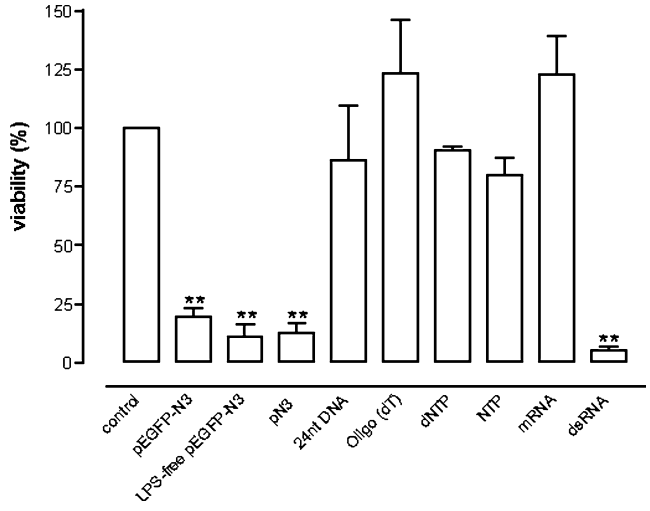

Fig. 2. Effect of nucleofection of various nucleic acids on the viability of J774A.1 macrophages as determined by neutral red viability assays. In each experiment, 10^6 macrophages were pulsed with one of the following nucleic acids (1 μg each): pEGFP-N3 plasmid ($n = 8$), LPS-free pEGFP-N3 ($n = 2$), pN3 plasmid ($n = 2$), 24 nt double-stranded oligonucleotides ($n = 5$), single-stranded 20 nucleotides oligo(dT) ($n = 3$), 50 nmol dNTP ($n = 2$), 50 nmol NTP ($n = 2$), eGFP mRNA ($n = 5$), and double-stranded (ds) eGFP RNA ($n = 3$). $^{**}p < 0.01$ vs. T20 control pulse ($n = 8$), ANOVA followed by the Dunnett test. Data are expressed as means ± SE of the mean.

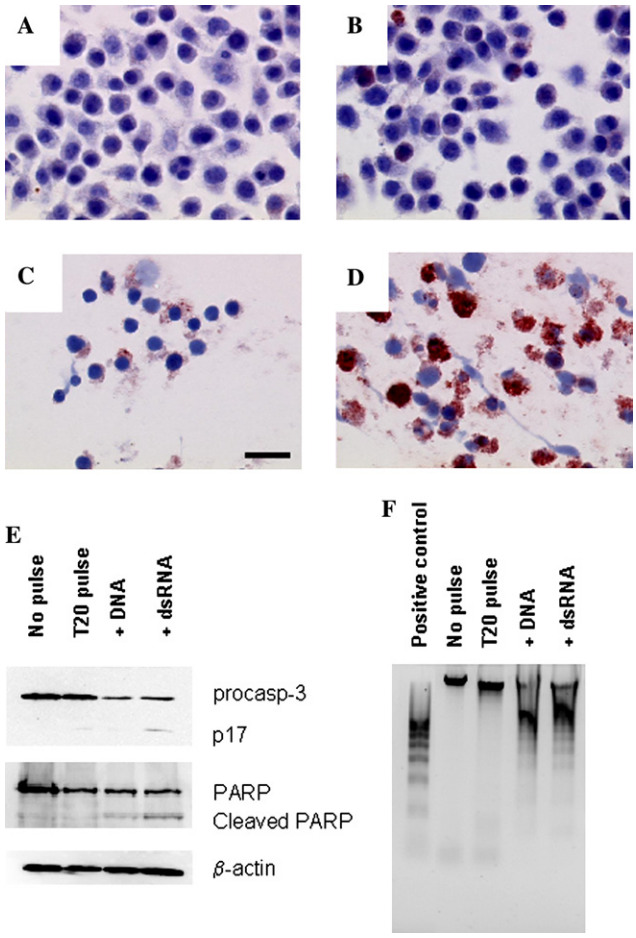

Fig. 3. Characterisation of cell death eighteen hours after nucleofection of J774A.1 macrophages. (A–D) Immunocytochemical detection of cleaved caspase-3 in unpulsed macrophages (A), pulsed cells without DNA (B), cells pulsed with 1 μg pEGFP-N3 plasmid DNA (C), and cells pulsed with 1 μg dsRNA (D). Scale bar = 25 μm. (E) Western blot analysis of caspase-3 and PARP-1 in J774A.1 macrophages. β-Actin was used to demonstrate equal loading. (F) DNA laddering in J774A.1 macrophages after nucleofection with DNA or dsRNA. U937 monocytes, treated with 50 μM etoposide for 4 h, were used as positive control (lane 1). Data are representative of three experiments.

fragmentation were used. Although we clearly showed phosphatidylserine externalisation (annexin V staining), internucleosomal DNA fragmentation as well as cleavage of caspase-3 and PARP-1 were limited after DNA transfection of the macrophages. This cell death phenotype was also observed by other groups after DNA transfection of macrophages and monocytic cell lines using standard electroporation [7,8]. The limited cleavage of caspase-3 and partial DNA fragmentation can be explained by the fact that the number of copies introduced into the macrophages determines the strength of the apoptotic stimulus. Uptake of a variable number of copies may be responsible for the reported large variation in eGFP expression levels among plasmid DNA-transfected cells [2]. Since apoptosis was more pronounced after dsRNA transfection, it is conceivable that macrophages take up dsRNA more easily than plasmid DNA due to its smaller size (737 bp [eGFP dsRNA] vs. 4700 bp [plasmid DNA encoding eGFP]) resulting in uptake of a higher number of dsRNA copies and more substantial cleavage of caspase-3 and PARP-1. This response of the macrophages is comparable with the normal defence mechanism of mammalian cells after infection with viruses containing dsRNA [9].

mRNA nucleofection proved to be superior to DNA transfections because mRNA is a normal constituent of the cell and thus less toxic. This finding is in accordance with previous reports showing that expression after mRNA transfer by electroporation was markedly improved as compared to DNA transfection [2,10]. Moreover, RNA lacks the potential to integrate into the host genome thereby obviating safety concerns (e.g., insertional mutagenesis) which is an important advantage during clinical gene therapy trials [11,12]. However, RNA has a short cellular half-life so that expression of the gene of interest is not guaranteed for a prolonged period of time. Nevertheless, recent studies have shown that gene expression after mRNA transfection may last for several days [10].

In conclusion, J774A.1 macrophages can be efficiently transfected with mRNA using nucleofector technology. However, introduction of DNA or dsRNA into macrophages leads to apoptosis. Hence, nucleofection with mRNA is a new high-speed transfection method for macrophages, which are hard-to-transfect, and enables a wide range of applications in this type of cells.

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