Nocodazole Treatment of CV-1 Cells Enhances Nuclear/Perinuclear Accumulation of Lipid-DNA Complexes and Increases Gene Expression

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INTRODUCTION

A major challenge to the successful implementation of gene therapy is the delivery of DNA to the nucleus of the target cell, despite the numerous cellular barriers that exist to circumvent this process. One major non-viral delivery strategy for genes involves complexation of the DNA with a mixture of cationic and neutral lipids to form lipid-DNA complexes which are thought to enter cells by endocytosis (1,2). Once endocytosed, lipid-DNA complexes must exit the endosomal/lysosomal pathway to be routed to the nucleus. A major contributor to membrane trafficking in the endosomal/lysosomal pathway is the cellular microtubule (MT) array, which forms a series of tracks that support the motor-driven movement of endosomes to lysosomes (3,4). In this study, we explore the effects of two MT-targeted agents, nocodazole and taxol, on the subcellular distribution and persistence of lipid-DNA complexes.

MATERIALS AND METHODS

Formulation of Lipid-DNA Complexes

Liposomes were formulated by the Valentis Bioprocessing Department at a 1:1 molar ratio of cationic lipid:co-lipid with the cationic lipid, 1-[2-[9-(Z)-octadecenoyloxy]]-2-[8](Z)-heptadecenyl]-3-[hydroxyethyl]imidazolinium chloride (DOTIM) (Sigma) (Megabios patent numbers 5,705,655 and 5,736,395), remaining constant in both formulations, and differing only with its corresponding co-lipid, 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) or Diphytanoyl Phosphoethanolamine (Diphytanoyl PE) (Avanti Polar Lipids, Alabaster, AL). The final cationic lipid concentration of the liposomes was 20 mM. Liposomes were complexed either to fluorescently-labeled vector (Rhodamine labeled CAT vector – expression inactive; purified and rhodamine-labeled by the Valentis Bioprocessing Department) or to a Valentis expression vector for luciferase, with the appropriate cationic liposome formulations at a 1:8 ratio of mg DNA/μmol cationic lipid. DNA was added to liposomes to make a final DNA: liposome complex concentration of 0.25 mg/ml, making the final net charge of the complex positive. Transfection with lipid-DNA constructs was at 1.25 μg/ml.

Cell Culture and Treatments

CV-1 cells were maintained in MEME containing 10% FBS and penicillin/streptomycin and split at confluence using trypsin-EDTA. For confocal fluorescence microscopy, cells were cultured in phenol red-free MEM containing 10% FBS, 10 mM HEPES and penicillin/streptomycin. Cell culture reagents were obtained from Gibco-Life Technologies. For all assays, taxol (4 μM, LC Laboratories, Woburn MA) or nocodazole (6.6 μM, Sigma) were added to the cells for 60 min at 37°C in a 5% CO_2 incubator prior to the addition of lipid-DNA complexes.

Confocal Fluorescence Microscopy

CV-1 cells were exposed to rhodamine-labeled lipid-DNA complexes for 15 min or 2 hrs, fixed in 4% paraformaldehyde, quenched with 50 mM NH_4Cl and mounted in Prolong Antifade mounting medium (Molecular Probes, Eugene OR). Slides were viewed with a Nikon PCM Confocal System equipped with filters for FITC and TRITC fluorescence attached to a Nikon TE300 Quantum upright microscope.

Luciferase Assay

Lipid-DNA complexes containing the luciferase reporter gene were incubated with the cells for 4 hrs. 24 hrs following their removal, cells were detached by trypsinization, pelleted and flash frozen at −80 °C. For analysis of luciferase activity, cell pellets were resuspended in 200–300 μl lysis buffer (0.1M Tris base, 0.05% Triton X-100, 0.002 M EDTA, pH 7.7) and centrifuged at 14,000 rpm for 4 min at room temperature to obtain cleared cell lysates. Lysates were loaded directly into 96-well plates for luciferase activity measurements. Firefly luciferase (R&D Systems, Minneapolis, MN) was utilized to obtain a standard curve. The Promega (Madison, WI) luciferase assay system was used to determine the amount of luciferase activity in the samples according to the manufacturer's instructions. Luciferase activity was normalized to the protein content of the pellets as measured using the BCA assay, with bovine serum albumin (Roche Molecular Biochemicals, Indianapolis, IN) as standard protein.

Statistical Analysis

Luciferase activity data were analyzed using a paired t-test with $p \leq 0.05$.

RESULTS

Nocodazole Enhances the Nuclear/Perinuclear Recovery of Rhodamine-Labeled DNA

The cellular distribution of rhodamine-labeled DNA encased in two different cationic liposomes (DOTIM: Diphytanoyl PE and DOTIM:DOPE) was analyzed in control, taxol-treated or nocodazole-treated CV-1 cells. Similar to previously published work (5), nocodazole and taxol elic-

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ited distinct effects on the MT array. Nocodazole resulted in a modest loss of MT polymer during the 60 min pretreatment, while taxol increased MT polymer content and accumulation of MT bundles within the same time course (data not shown).

For the purposes of qualitative (Figure 1) and quantitative (Table I) confocal microscopy analysis, the cell was subdivided into three roughly equivalent cellular areas: the plasma membrane region (cell periphery), the intermediate cytoplasm and the perinuclear/nuclear region as shown in Figure 2. The perinuclear/nuclear region encompasses both the nucleus and the area of cytoplasm immediately surrounding the nucleus. Figure 1A shows the distribution of rhodamine-labeled DNA encased in the DOTIM:Diphytanoyl PE liposome in control cells after 15 min of exposure. Almost all rhodamine-tagged DNA was recovered at the cell periphery. After 2 hrs, more fluorescent DNA was present in the cell interior, with most rhodamine-labeled DNA associated with the intermediate region of the cell (Figure 1B). Taxol treatment (4 μM, 60 min) prior to incubation with the construct for 15 min (Figure 1C) or 2 hrs (Figure 1D) did not alter this distribution pattern. Nocodazole treatment (6.6 μM, 60 min) revealed a comparable distribution of rhodamine-labeled DNA after 15 min of exposure (Figure 1E), but the distribution of rhodamine-labeled DNA after 2 hrs of exposure was notably shifted with the majority recovered on and around the nucleus (Figure 1F). Increased fluorescence recovery was detected both in the area overlying the nucleus itself and in the area surrounding the nucleus (perinuclear region). Because CV-1 cells are extremely flat, confocal microscopy also provided insufficient resolution to distinguish whether the

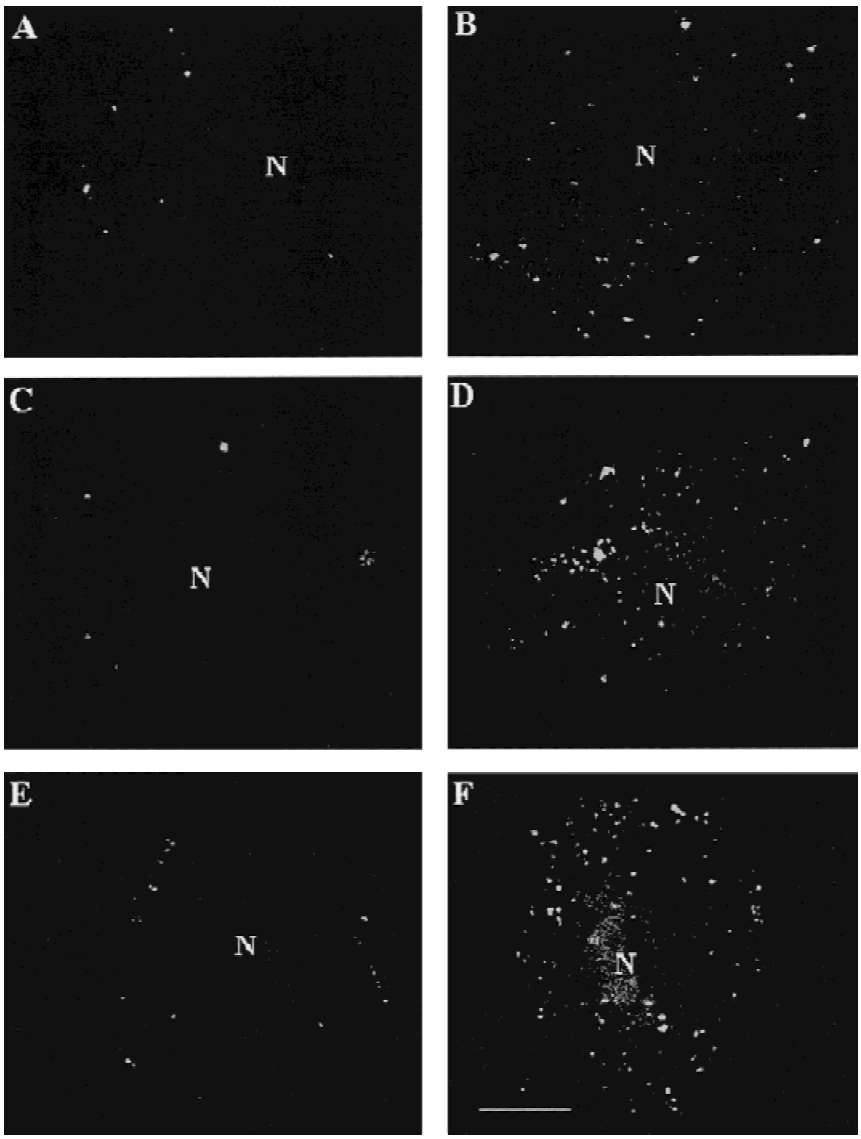

Fig. 1. Effects of MT-targeted drugs on distribution of rhodamine-labeled DNA packaged in DOTIM:Diphytanoyl PE. A., C. and E. show the distribution of rhodamine-labeled DNA packaged in DOTIM:Diphytanoyl PE after 15 min while B., D. and F. show its distribution after 2 hrs. A. and B., control CV-1 cells. C. and D., taxol-treated (4 μM, 60 min pretreatment) CV-1 cells and E. and F., nocodazole-treated (6.6 μM nocodazole, 60 min pretreatment). In C.-F., the drug was maintained in the medium during the incubations with lipid-DNA constructs. Bar, 15 μm.

Table I. Cellular Distribution of Rhodamine-labled DNA Encased in DOTIM:Diphytanoyl PE or DOTIM:DOPE within CV-1 Cells[a]

	DOTIM:Diphytanoyl PE				DOTIM:DOPE			
Treatments	Plasma membrane	Intermediate	Perinuclear/ nuclear	No labeling	Plasma membrane	Intermediate	Perinuclear/ nuclear	No labeling
Control, 15 min	85 ± 3	12 ± 3	N.D.	3 ± 1	86 ± 4	10 ± 3	N.D.	4 ± 1
Control, 2 hrs	2 ± 1	73 ± 1	25 ± 2	N.D.	8 ± 7	74 ± 8	18 ± 3	N.D.
Taxol, 15 min	83 ± 4	16 ± 4	N.D.	1 ± 1	87 ± 3	12 ± 3	N.D.	1 ± 0.3
Taxol, 2 hrs	4 ± 3	71 ± 4	25 ± 6	N.D.	N.D.	78 ± 6	22 ± 6	N.D.
Noc, 15 min	85 ± 3	11 ± 2	N.D.	4 ± 1	76 ± 1	22 ± 2	1 ± 1	1 ± 1
Noc, 2 hrs	4 ± 4	57 ± 10	39 ± 7	N.D.	5 ± 4	53 ± 8	42 ± 3	N.D.

[a] Cells were pretreated for 60 min with Taxol (4 μM) or Nocodazole (Noc, 6.6 μM) before addition of rhodamine-labeled DNA encased in either DOTIM:Diphytanoyl PE or DOTIM:DOPE for the indicated times. The distribution of fluorescence in the cell periphery, in the intermediate region and in the perinuclear/nuclear region as identified morphologically in Figure 2 was measured in 209–628 individual cells per experiment across three separate experiments. Cells were selected randomly. Nuclear regions could usually be detected morphologically in fluorescently labeled cells but the identification of the nucleus was confirmed whenever necessary by differential interference contrast microscopy imaging of the same field. Values are expressed as the percentage of the total cell number in each preparation that fell within each category. N.D., not detected.

fluorescence associated with lipid-DNA constructs overlying the nucleus was recovered within the nucleus or just outside the nuclear envelope. We therefore describe this effect of nocodazole as an enhancement in the recovery of lipid-DNA in the nuclear/perinuclear region. In addition to the enhanced nuclear/perinuclear recovery of the rhodamine-labeled DNA, nocodazole-treated cells appeared more intensely labeled with rhodamine-DNA after 2 hrs (Figure 1F) relative to controls (Figure 1B). Nocodazole but not taxol elicited a comparable increase in nuclear/perinuclear recovery of rhodamine-labeled DNA encased in DOTIM:DOPE liposomes after 2 hrs (data not shown).

A quantitative summary of the distribution of rhodamine-labeled DNA encased in either DOTIM:Diphytanoyl PE or DOTIM:DOPE is shown in Table I. The increased recovery of rhodamine-labeled DNA in perinuclear/nuclear regions in nocodazole-treated cells after 2 hrs is evident with both constructs, resulting in a ~1.5–2-fold increase in the number of cells exhibiting this labeling pattern.

Nocodazole Increases Luciferase Expression in Transfected CV-1 Cells

Control and treated CV-1 cells were incubated with the same lipid-DNA constructs containing a luciferase-reporter gene instead of rhodamine-labeled DNA for 4 hrs, cultured for 24 hrs, and analyzed for luciferase expression. Previous studies have shown that expression of luciferase is maximal after 24 hrs (data not shown). Figure 3 shows luciferase expression across several preparations of CV-1 cells transfected with the luciferase reporter gene packaged in DOTIM: Diphytanoyl PE (Figure 3A) or DOTIM:DOPE (Figure 3B).

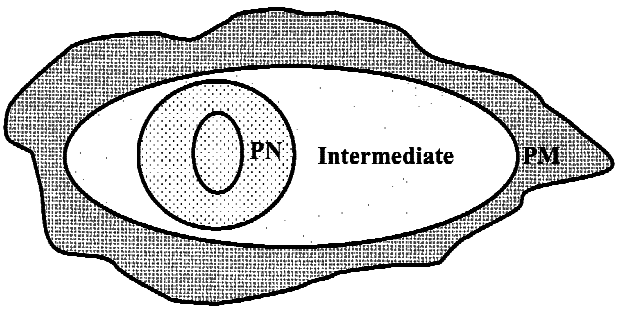

Fig. 2. Cellular regions considered in analysis of fluorescent lipid-DNA construct uptake. This diagram shows the three cellular regions analyzed in Table 1: Perinuclear/nuclear (PN), Intermediate and Plasma Membrane (PM).

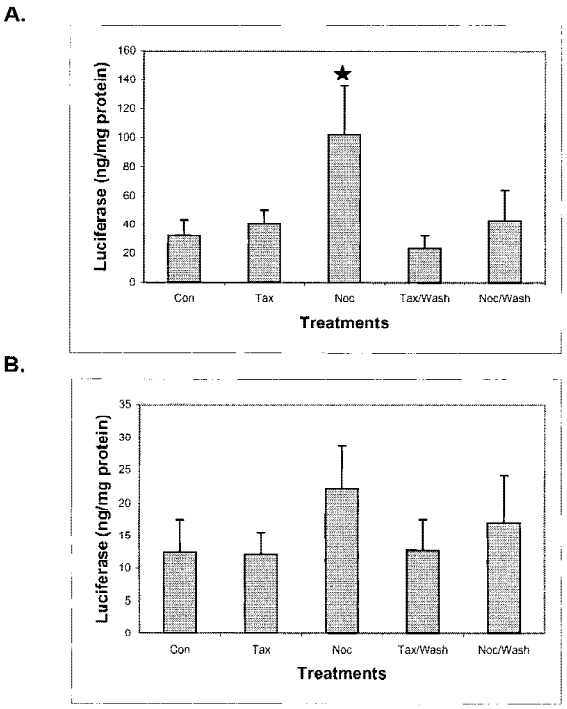

Fig. 3. Effects of MT-targeted drugs on gene expression. A. Luciferase expression from DNA packaged in DOTIM:Diphytanoyl PE. B. Luciferase expression from DNA packaged in DOTIM:DOPE. Con, control; Noc, nocodazole; Tax, taxol. Nocodazole (6.6 μM) and taxol (4 μM) were added 60 min prior to addition of lipid-DNA constructs and cells were incubated for another 4 hrs prior to replacement of this medium with fresh medium. Noc/Wash and Tax/Wash refer to the washout of drugs after the 60 min pretreatment, before addition of lipid-DNA constructs for 4 hrs. Luciferase expression was assessed 24 hrs after removal of lipid-DNA constructs. Data are shown as mean ± s.e.m. from 3 preparations and are expressed as ng luciferase/mg protein, *significant at $p \leq 0.05$.

Nocodazole markedly increased the expression of the luciferase gene encased in either DOTIM:Diphytanoyl PE and DOTIM:DOPE, while taxol had no detectable effect. Washout of the drug after nocodazole pretreatment but before addition of the lipid-DNA complexes (Noc/Wash) eliminated most of the increased increment of luciferase expression, showing that the continuous presence of this reversible agent was necessary for the increase.

DISCUSSION

Here, we demonstrate that nocodazole enhances the nuclear/perinuclear targeting of lipid-DNA complexes in parallel with increased gene expression of the transfected DNA. Because nocodazole targets the MT array and is known to uncouple endosome to lysosome traffic (5), one explanation for its effects is via changes in MT-dependent vesicle transport. If following endocytosis, lipid-DNA complexes are normally routed via MT-dependent vesicle transport to the lysosomes for degradation, inhibition of this routing step by nocodazole could allow rapid exit of lipid-DNA complexes from endosomes before their degradation in lysosomes. Consistent with this, a previous study has found that colchicine enhances the persistence of DNA introduced via receptor-mediated drug targeting in the liver (6).

The lack of enhancement by taxol was somewhat surprising since it also affects cellular MTs. However, taxol promotes MT accumulation rather than MT loss, an effect that may have different consequences to MT-dependent vesicle transport. In fact, previous work comparing the effects of taxol and nocodazole on receptor-mediated endocytosis of transferrin in CV-1 cells has revealed distinct differences in the dynamics of transferrin movement through the endocytic pathway (5). Alternatively, taxol elicits additional signaling effects including activation of kinases and induction of apoptosis (for examples see 7, 8) which may confound otherwise beneficial effects on traffic of internalized DNA. Although nocodazole and taxol can modulate the MT array in different ways, they both affect MTs. It is therefore conceivable that the beneficial effects of nocodazole on gene targeting and persistence may occur through a MT-independent mechanism.

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