

Effect of Vinblastine on Transfection: Influence of Cell Types, Cationic Lipids and Promoters

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Abstract: As previously shown, vinblastine, when incorporated into a cationic lipid prior to generation of lipoplexes, increases by ~30-fold the extent of transfection of p -Gal with a cytomegalovirus promoter (pCMV- -Gal) to vascular smooth muscle cells (VSMC) by 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC)-pCMV- -Gal complexes. To test if this increase is limited to VSMC and EDOPC, or is general, we examined three other cell types, human umbilical artery endothelial cells (HUAEC), baby hamster kidney (BHK) cells and 293 cells derived from human kidney, as well as a different cationic lipid, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). In addition, to determine the contribution of the NF- B transcription factor to the vinblastine effect, pCMV was replaced with a smooth muscle -actin gene promoter, SMGA, which, unlike pCMV, does not respond to NF- B. It was found that on all cell types we tested, the transfection efficiency increased with vinblastine incorporation; however, the magnitude depended greatly on the cell type, e.g. whereas the transfection of VSMC increased ~30-fold, that of 293 cells increased only ~2-fold. The cationic phospholipid could be replaced with DOTAP with no loss of effect. In contrast, the promoter was critical and the stimulation was lost if pCMV was replaced with pSMGA. It is concluded that the positive effect of vinblastine on transfection is general and the stimulation of the transcription factor NF- B is involved in this action. The activation of NF- B by anti-microtubule agents should thus allow for transfection of specific cell types by vinblastine lipoplexes.

Keywords: Vinblastine, transfection, smooth muscle -actin gene promoter, NF- B, intracellular transport.

INTRODUCTION

In comparison to viral-based carriers, lipid-based¹ non-viral vectors have the advantage of low immunogenicity, safety, ability to package large DNA molecules, and ease of preparation [1]. Although advances in formulation of lipoplexes have led to significant improvements, lipid-mediated gene delivery has not yet approached the high efficiency of viruses. There are several barriers to gene transfer by a cationic lipid. First, DNA-cationic lipid complexes must enter the cell, usually by endocytosis. Second, DNA must escape endosomes prior to their fusion with lysosomes. Third, DNA must enter the nucleus. Finally, DNA must be transcribed efficiently and appropriately. Our previous work [2] has shown that vinblastine (VB), when incorporated into cationic lipid, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EDOPC), prior to generation of lipoplexes, increases by ~30-fold the extent of transfection of vascular smooth muscle cells (VSMC), and that two mechanisms are involved: (i) inhibition of transport of lipoplexes to lysosomes; (ii) activation of transcription (via NF- B). Since these constitute two of the four barriers that lipoplexes have to overcome prior to DNA expression, we supposed that this formulation should not be

limited to VSMC, but could also operate in other cell types to stimulate gene transfection. To test this hypothesis, we examined other cell types, human umbilical artery endothelial cells (HUAEC), baby hamster kidney (BHK) cells and 293 cells derived from human kidney. In addition, to determine the influence of cationic lipid, a different cationic lipid, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), was used to replace EDOPC.

MATERIALS AND METHODS

Vinblastine and DOTAP were purchased from Sigma (St. Louis, MO) and Avanti Polar-Lipids, Inc (Alabaster, AL), respectively. EDOPC was synthesized according to MacDonald *et al.* [3]. CMV- -galactosidase plasmid (pCMV- -Gal) was purchased from Clontech Laboratories Inc. (Palo Alto, CA) and propagated and purified by Bayou Biolabs (Harahan, LA). SMGA- -galactosidase plasmid contains the -2294 bp SMGA promoter (-2294 to +25) with respect to the transcriptional start site; Zimmer *et al.* [4] cloned into the pGAL basic plasmid (Promega) to drive galactosidase expression.

Primary vascular smooth muscle cells were obtained by removal of the thoracoabdominal aortas of Wistar rats, which were then stripped of endothelium and adventitia [5]. Medial VSMCs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 20% fetal bovine serum (FBS). BHK cells and 293 cells were obtained from ATCC (Rockville, MD) and maintained as recommended. HUAEC were obtained from BioWhittaker, Inc (Walkersville, MD)

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¹ Although the term "lipid" is commonly used to describe these agents, none of them correspond to natural products and hence they are properly termed "lipoids".

and cultured in EGM-2 MV BulletKit (BioWhittaker, Inc, Walkersville, MD).

The cells were seeded in 96-well plates at 24 h before transfection at densities to give about 80% confluence at the time of transfection. Cationic lipids with or without vinblastine (for amounts, see figure legends) were suspended in Dulbecco's PBS (D-PBS) at 1 mg/ml to form liposomes. Liposomes and plasmid DNA were diluted in serum-free cell culture medium to 60 µg/ml for lipid and to 20 µg/ml for DNA, and liposomes were pipetted into an equal volume of plasmid DNA solution at a 3:1 weight ratio and mixed gently. The resultant DNA-lipid complexes were incubated at room temperature for 15 min and then 50 µl per well (0.5 µg DNA/well) were added to the cells that were either in medium lacking serum or medium containing 20% serum. At 3 h after addition of DNA-lipid complexes, the cells were washed with D-PBS and fresh medium containing serum was added.

Cells were assayed for β -galactosidase activity 24 h after transfection using a microplate fluorometric assay [6],

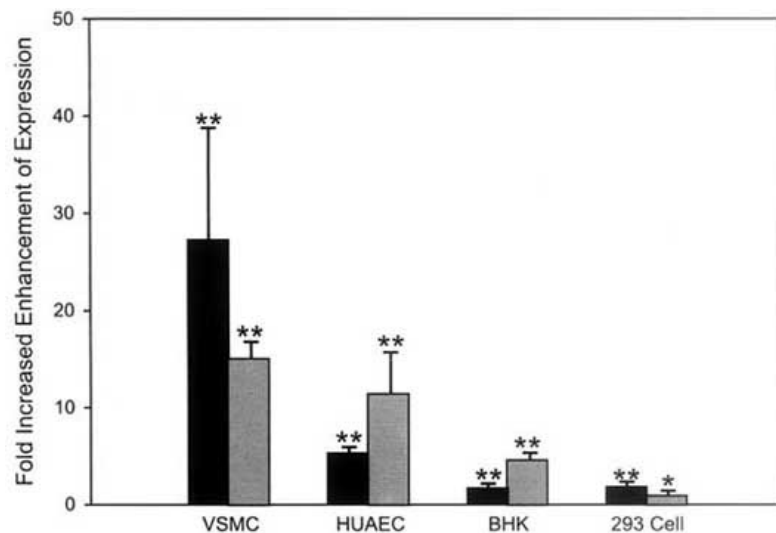
modified by inclusion of a heating step (50°C, 45 min) to inactivate endogenous enzyme activity. Following aspiration of the medium from each well, the cells were washed once with D-PBS and then lysed by addition of 100 µl lysis buffer (0.03% Triton X-100 in 100 mM HEPES, pH 7.8, containing 1 mM MgSO₄, 10 mM KCl). The plates were placed at 50°C for 45 min and then allowed to cool to room temperature. 10 µl 100 µM FDG (fluorescein di- β -D-galactopyranoside) were added into each well. Fluorescence was measured with a microplate fluorimeter (Model 7620, Cambridge Technology Inc.) after incubation at 37°C for 3 h.

X-gal staining was used to determine the number of transfected cells histochemically according to the procedure provided by Invitrogen Life Technologies (Carlsbad, CA).

RESULTS AND DISCUSSION

Fig. 1 shows the effect of vinblastine on the transfection of HUAEC, BHK and 293 cells with EDOPC-pCMV- β -Gal complexes. Compared to the transfection of VSMC, which

A.



B. Transfection Efficiency as Measured by X-gal Staining

cell type	no serum no VB	with serum no VB	no serum with VB	with serum with VB
VSMC	2%	1%	15%	5%
HUAEC	0.5%	<0.5%	2%	3%
BHK	20%	20%	35%	40%
293 cells	65%	20%	80%	50%

Fig. (1). Effect of vinblastine on the transfection of VSMC, HUAEC, BHK and 293 cells with EDOPC-pCMV- β -Gal complexes. Vinblastine was incorporated in EDOPC at the ratio of 1: 100 (by weight) prior to generation of liposomes and formation of lipoplexes and then the cells were treated with the resulting lipoplexes. Black and gray bars denote that serum was absent or present during the incubation with transfection complexes, respectively. The figure (A) shows increased transgene expression relative to absence of vinblastine and serum. Data represent the means \pm S.D. of quadruplicate wells in one typical experiment. The experiment was repeated twice with consistent results. **Compared with no vinblastine, $P < 0.01$. *Compared with no vinblastine, $P > 0.05$. The table (B) shows the transfection efficiency as measured by X-gal staining.

increased 27- and 14-fold in the absence and presence of serum respectively, the transfection of HUAEC, BHK and 293 cells increased 5- and 11-, 2- and 4-, 2- and 1-fold in the absence and presence of serum respectively, relative to the absence of vinblastine and serum.

Fig. 2 illustrates that when DOTAP, another kind of cationic amphipath, was used as the transgene vector, the inclusion of vinblastine also produced a significant increase in transfection, i.e., an 8- and 3-fold increase in the absence and presence of serum, respectively.

Pyrrolidinedithiocarbamate (PDTC) [7] and N -p-tosyl-L-lysine chloromethyl ketone (TLCK) [8, 9], inhibitors of NF- κ B activation, were found to inhibit vinblastine stimulation of transfection 85% and 66%, respectively [2]. Consequently, it was presumed that the action of vinblastine was related to NF- κ B transcription pathway. Here we used another, more direct, method to test this hypothesis. CMV-IEP- β -Gal was replaced with SMGA- β -Gal (smooth muscle α -actin gene promoter), which does not respond to NF- κ B [10]. Fig. 3 shows that transfection of this plasmid is not stimulated by vinblastine at any concentration examined.

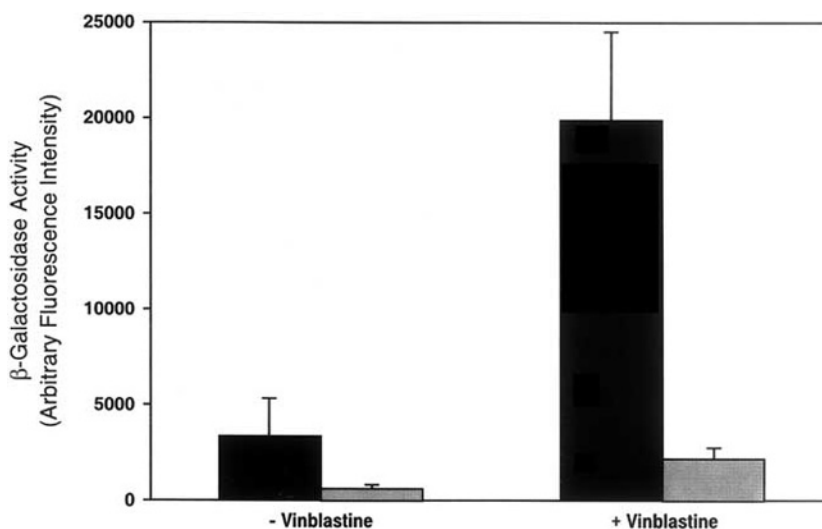


Fig. (2). Effect of vinblastine on the transfection of VSMC with DOTAP-pCMV- β -Gal complexes. Vinblastine was incorporated in DOTAP at the ratio of 1: 100 (by weight) prior to generation of liposomes and formation of lipoplexes and then the cells were treated with resulting lipoplexes. Black and gray bars denote that serum was absent or present during the incubation with transfection complexes, respectively. Data represent the means \pm S.D. of quadruplicate wells in one typical experiment. The experiment was repeated twice with consistent results. \sim 2000 fluorescence units corresponded to 0.1 milliunit of β -galactosidase.

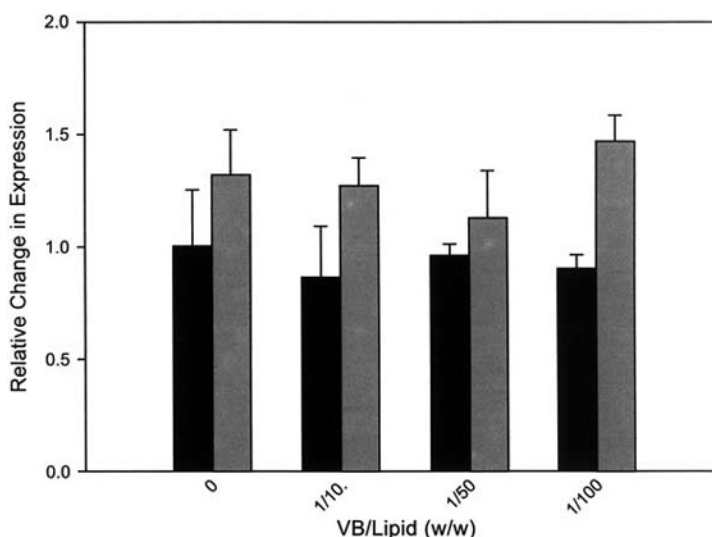


Fig. (3). Effect of vinblastine on the transfection of VSMC with EDOPC-pSMGA- β -Gal, under conditions where vinblastine was incorporated in the lipid prior to generation of liposomes and formation of lipoplexes, as a function of the vinblastine concentration, with and without serum. The cells were treated with DNA-lipid complexes for 3 h. Black and gray bars denote that serum was absent or present, respectively, during the 3 h incubation with transfection complexes. The figure shows the fluorescence intensity relative to absence of vinblastine and serum during transfection treatment. Data represent the means \pm S.D. of quadruplicate wells in one typical experiment. The experiment was repeated twice with consistent results.

Transfection efficiency increased with vinblastine incorporation for all cell types we tested; however, the magnitude of the effect depended greatly on the cell type. The type of cationic lipid does not seem to be critical. This suggests that the stimulatory effect of vinblastine on gene transfection is quite general, but the promoter does appear to be critical because the stimulation is absent if pCMV is replaced with pSMGA. The CMV promoter has been reported to have four repeats of the NF- κ B binding sequences [11], which are absent from the smooth muscle α -actin gene promoter [10]. Thus, upon activation, NF- κ B may bind to its corresponding sites in plasmid DNA, and then the NF- κ B-associated plasmid DNA is translocated into nucleus through the nuclear pore complex. Consistent with such action is the report from Mesika *et al.* [12] that NF- κ B assisted the import of plasmid DNA into the nuclei of mammalian cells *in vitro*. It is also possible that NF- κ B participates in the activation of transcription of plasmid DNA [13, 14], for there is evidence [15] that NF- κ B not only regulates the expression of endogenous genes, but also of some exogenous genes. High multiplicity infection with a replication-deficient adenoviral vector activates NF- κ B, which leads to augmented gene expression from the CMV-IEP (human cytomegalovirus immediate-early promoter). The link between the endogenous NF- κ B activation and exogenous gene-increased expression may be the CMV-IEP element, in which case the promoter is critical for the stimulatory effect of vinblastine on gene transfection.

How do microtubule depolymerizing agents lead to activation of NF- κ B? As with other inducers of NF- κ B, such as TNF- α , microtubule-depolymerizing agents lead to I κ B degradation [14]. The critical step leading to I κ B degradation may be its phosphorylation. Two protein kinases, which phosphorylate I κ B, MAP kinase [16] and protein kinase A [17], have been shown to be activated by colchicine. Our result that the serine protease inhibitor TLCK inhibited vinblastine stimulation of transfection 66% also suggests the involvement of protein kinase [2]. Whatever the mechanism of NF- κ B activation, the microtubule depolymerizing agent-gene combination in one formulation provides a new strategy to enhance transgene expression. Vinblastine is well suited to this function in

cells responsive NF- κ B because it is hydrophobic enough to be retained by lipids, but it would not be surprising if a number of other hydrophobic, transcription-activating drugs are available that could be used to good effect in cells that are unresponsive to NF- κ B.

The present result supports an involvement of NF- κ B but not of intracellular transport in the effect of vinblastine. The latter is unexpected and suggests that the effect of lipoplex-delivered vinblastine on endocytosis and trafficking is complex and requires further study.

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REFERENCES

- [1] Zabner, J. *Adv. Drug Deliv. Rev.*, **1997**, 27, 17.
- [2] Wang, L.; MacDonald, R.C. *Mol. Ther.*, **2004**, 9, 729.
- [3] MacDonald, R.C.; Rakhmanova, V.A.; Choi, K.L.; Rosenzweig, H.S.; Lahiri, M.K. *J. Pharm. Sci.*, **1999**, 88, 896.
- [4] Zimmer, W.E.; Browning, C.L.; Kovacs, A.M. *Dev. Biol.*, **1991**, 175, 121.
- [5] Crowley, S.T.; Dempsey, E.C.; Horwitz, K.B.; Horwitz, L.D. *Circulation*, **1994**, 90, 1908.
- [6] Rakhmanova, V.A.; MacDonald, R.C. *Anal. Biochem.*, **1998**, 257, 234.
- [7] Schreck, R.; Rieber, P.; Baeuerle, P.A. *EMBO J.*, **1991**, 10, 2247.
- [8] Fan, C.; Li, Q.; Ross, D.; Engelhardt, J.F. *J. Biol. Chem.*, **2003**, 278, 2072.
- [9] Henkel, T.; Machleidt, T.; Alkalay, I.; Kronke, M.; Ben Neriah, Y.; Baeuerle, P.A. *Nature*, **1993**, 365, 182.
- [10] Carson, J.A.; Fillmore, R.A.; Schwartz, R.J.; Zimmer, W.E. *J. Biol. Chem.*, **2000**, 275, 39061.
- [11] Assogba, B.D.; Choi, B.H.; Rho, H.M. *Virus Res.*, **2002**, 84, 171.
- [12] Mesika, A.; Grigoreva, I.; Zohar, M.; Reich, Z. *Mol. Ther.*, **2001**, 3, 653.
- [13] Sha, W.C.; Liou, H.C.; Tuomanen, E.I.; Baltimore, D. *Cell*, **1995**, 80, 321.
- [14] Rosette, C.; Karin, M. *J. Cell Biol.*, **1995**, 128, 1111.
- [15] Clesham, G.J.; Adam, P.J.; Proudfoot, D.; Flynn, P.D.; Efstathiou, S.; Weissberg, P.L. *Gene Ther.*, **1998**, 5, 174.
- [16] Shinoharagotoh, Y.; Nishida, E.; Hoshi, M.; Sakai, H. *Exp. Cell Res.*, **1991**, 193, 161.
- [17] Manie, S.; Schmidalliana, A.; Kubar, J.; Ferrua, B.; Rossi, B. *J. Biol. Chem.*, **1993**, 268, 13675.