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Effects of Lipofectamine 2000/siRNA Complexes on Autophagy in Hepatoma Cells

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Abstract

Lipofectamine 2000 is commonly used for siRNA transfections. However, few studies have examined cellular responses to this delivery system. The purpose of this study is to evaluate the effect of siRNA transfection using Lipofectamine 2000 on cellular autophagy. Huh7.5 cells, stably-transfected to express GFP-LC3, were treated with Lipofectamine 2000/negative control siRNA (NC siRNA) complexes. At different time points after treatment, cells were lysed and analyzed by immunoblotting and fluorescence spectroscopy. Cells were also observed using confocal microscopy. An increase of endogenous LC3 lipidation, GFP-LC3 fluorescence, and autophagosomal puncta was observed in cells treated with Lipofectamine 2000/NC siRNA complexes. The kinetics of the increase of GFP-LC3 fluorescence correlated with the concentration of NC siRNA transfected, where 50, 100, and 200 nM NC siRNA caused a significant increase at 72, 48 and 24 hours, respectively, after transfection. A similar effect on the GFP-LC3 signal was also observed for cells treated with Lipofectamine 2000 complexed with two other NC siRNAs. Lipofectamine 2000-mediated transport of NC siRNAs led to an increase of autophagosomes in a dose- and time-dependent manner. Thus, this effect on cells should be taken into consideration when using this approach for intracellular delivery of siRNA.

Keywords

autophagy; siRNA; Lipofectamine 2000; GFP-LC3; autophagosomes; siRNA delivery

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Introduction

Since the discovery of RNA interference (RNAi) [1], small interfering RNA (siRNA) has become an important and prominent research technology. Through the RNAi pathway, exogenously delivered siRNA cooperates with other cytosolic proteins to disrupt gene expression by degrading the target messenger RNA (mRNA) [2]. The ability to silence specific genes is a useful application to study gene function [3], determine molecular pathways [4], discover prospective drug targets, and develop pharmaceutical therapeutics [5]. Due to its negative charge and large size, intracellular uptake of siRNA is limited and requires a delivery moiety to aid its transport into the cells [6]. Lipofectamine 2000 is a commonly used reagent for siRNA delivery. This cationic lipid interacts with the negatively charged siRNA backbone to form a complex that enhances the oligonucleotide uptake [7]. In addition, since it is relatively efficient and widely applicable to various oligonucleotides, delivery using Lipofectamine 2000 has also been commonly used as a standard to compare the effectiveness of other novel siRNA delivery vehicles [8]. However, with the exception of concerns about cytotoxicity, a limited number of studies have been carried out to evaluate other inadvertent cell responses caused by Lipofectamine 2000/siRNA complexes.

The purpose of this study is to evaluate the cellular autophagic response to siRNA transfection using Lipofectamine 2000. Autophagy is a vital cellular process involving lysosomal-dependent intracellular degradation of proteins and organelles. It is highly regulated and plays an important role in cell growth, cell survival, immune defense and cellular homeostasis [9]. Among the different types of autophagy, the most commonly referred to is macroautophagy. Macroautophagy describes the collection of cytosolic components by double-membrane vesicles called autophagosomes that eventually fuse with lysosomes for degradation of cargo [10]. In this paper, the term “autophagy” refers to macroautophagy.

In this report, several distinct non-targeting, negative control siRNAs (NC siRNA), without any particular gene silencing effects, were complexed with Lipofectamine 2000 and evaluated for their cellular autophagic response. The studies were performed in Huh7.5 hepatoma cells expressing a fluorescent autophagosome marker, green fluorescent protein (GFP) fused to microtubule-associated protein 1, light chain 3 (LC3) (GFP-LC3). Autophagosome formation was determined by LC3 conversion, GFP-LC3 fluorescence quantification and confocal microscopy.

Materials and Methods

Cell culture and siRNA uptake

Huh7.5 hepatoma cells, transfected with pEGFP-LC3 followed by G418 (Invitrogen, Carlsbad, CA) selection to stably express GFP-LC3 fusion protein, had been described [11]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids and 2 mM L-glutamine (“complete medium”) (Invitrogen). As a positive control, cells were treated with 10 μ M MG-132 (Calbiochem, La Jolla, CA) for 24 h or 40 mM glucosamine (Sigma-Aldrich, St. Louis, MO) for 48 h to validate the mTOR-dependent or -independent autophagic response

of stably-transfected cells, respectively. Thereafter, for transient responses, cells were treated with NC siRNA ("NC siRNA 1" and "NC siRNA 2" were from Ambion, Foster City, CA, and "NC siRNA 3" was from Qiagen, Valencia, CA) using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, prior to treatment, Lipofectamine 2000/siRNA complexes were prepared in reduced serum medium, OptiMEM (Invitrogen), at the recommended ratio of 1 μ l Lipofectamine 2000 per 20 pmole siRNA. Cells were then treated for six hours before being replaced with complete medium for the desired duration. In this paper, Lipofectamine 2000/siRNA complex concentrations are defined by the siRNA concentration.

Western blotting

At the indicated treatment intervals, cells were washed with cold PBS, trypsinized and lysed with cell extraction buffer (Invitrogen) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) according to manufacturer's instruction. After protein quantification using Bicinchoninic Acid (BCA) Assay (Pierce, Rockford, IL), equal amount of cellular protein were subjected to electrophoresis in 15% SDS-PAGE gels followed by Western blotting against polyclonal rabbit anti-LC3 (MBL International, Woburn, MA) and monoclonal mouse anti-actin (Sigma-Aldrich) primary antibodies with corresponding HRP-conjugated secondary antibodies (Sigma-Aldrich). Immunoreactive bands were detected using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) and quantified by densitometry using Quantity One ® (Bio-Rad, Hercules, CA).

Quantification of GFP-LC3 fluorescence

After the designated treatment time intervals, cell lysates were collected, and transferred to a 96-well plate for fluorescence detection using a microplate reader (Tecan, Männedorf, Switzerland) with excitation at 485 nm and emission at 518 nm. Cell lysates were then quantified for protein content using BCA Assay. GFP-LC3 fluorescence was defined as relative fluorescence units (RFU) per mg of cell protein.

Confocal microscopy

Huh7.5 cells were fixed with 4% para-formaldehyde (Sigma) in PBS and then briefly incubated with 4', 6-diamidino-2-phenylindole (DAPI), dilactate dye (Invitrogen). Thereafter, cells were mounted using Prolong Antifade Kit (Invitrogen) according to manufacturer's instructions. Finally, coverslips were viewed using Nikon PCM Quantitative Measuring High-Performance Confocal System at the USC Research Center for Liver Diseases.

Statistical analysis

Data were presented as mean with error bars representing standard deviation and analyzed by Student's *t*-test. Results that yielded $p < 0.05$ were considered statistically significant, as indicated with an asterisk (*).

Results

Induction of LC3 Conversion by Lipofectamine 2000/NC siRNA Complexes

LC3-I is a soluble cytosolic protein that reacts with phosphatidylethanolamine (PE) upon autophagy activation. During autophagy, LC3-I is converted to a PE-conjugated form, LC3-II, which subsequently becomes membrane bound to an autophagosome. Therefore, the relative amount of LC3-II compared to non-treated control cells is considered an indicator of autophagosome accumulation [12]. Although LC3-II is larger in molecular weight than LC3-I, the PE-conjugated form migrates faster during the separation of these two endogenous proteins by electrophoresis due to its high hydrophobicity [12]. Treating stably-transfected Huh7.5 cells with 10 μ M MG-132, a known activator of autophagy [13], resulted in a greater amount of LC3-II compared with non-treated cells (Fig. 1a). This result validated the use of this cell line and Western blot method to study autophagy responses. After confirming the autophagic response of the Huh7.5 cells using the appropriate controls, the cells were treated with Lipofectamine 2000/NC siRNA 1 complexes. Our results showed that, 48 h after treatment, the amount of LC3-II increased by more than 2-fold following treatment with Lipofectamine 2000 alone (10 μ g/ml, equivalent to the Lipofectamine 2000 concentration in 200 nM Lipofectamine 2000/NC siRNA 1 complex) (Fig. 1b, Lane 2), while the amount of LC3-II following treatment with NC siRNA 1 alone (200 nM, Lane 3) was slightly increased (1.1-fold) compared to the non-treated control (Lane 1). Interestingly, a dose-dependent increase of LC3-II amount following treatment with 50-200 nM Lipofectamine 2000/NC siRNA 1 complexes was observed, with approximately a 2- and 5-fold increase for the 100 and 200 nM Lipofectamine 2000/NC siRNA 1 complexes, respectively (Lanes 4-6). Therefore, treatments of Lipofectamine 2000 alone, or with 100 and 200 nM Lipofectamine 2000/NC siRNA 1 complexes were able to increase the number of autophagosomes.

Enhancement of GFP-LC3 Fluorescence by Lipofectamine 2000/NC siRNA Complexes

Huh7.5 cells carrying GFP-LC3 fusion protein were used to evaluate the amount of GFP-LC3 fluorescence after treatment with Lipofectamine 2000/NC siRNA 1 complexes. GFP-LC3 is most commonly used for the visualization of autophagosomes during autophagy [14, 15], but more recently, flow cytometry-based applications have been used to quantify autophagic activity [16, 17]. We established a fluorescence-based assay to determine and quantify total GFP-LC3 expression levels, which correlate with the amount of autophagosome present. Cells treated with 10 μ M MG-132 or 40 mM glucosamine exhibited approximately 6- and 3-fold more fluorescence than non-treated cells, respectively (Fig. 2a). Therefore, this stably-transfected cell line behaved as expected in response to mTOR-dependent (MG-132 treated) [18, 19] and mTOR-independent (glucosamine treated) [20] autophagy activation treatments. When treated with different concentrations of Lipofectamine 2000/NC siRNA 1, no significant difference of GFP-LC3 levels was observed compared with the non-treated control group at 6 h after treatment (Fig. 2b). At 24 h, only the highest concentration of Lipofectamine 2000/NC siRNA 1 complex, 200 nM, significantly increased GFP-LC3 production by 22% (Fig. 2b). At 48 h, both 100 and 200 nM Lipofectamine 2000/NC siRNA 1 complex significantly enhanced GFP-LC3 expression by 29 and 54%, respectively (Fig. 2b). Incubation for 72 h led to the greatest increase for all three concentrations of Lipofectamine 2000/NC siRNA 1 complex, 50, 100 and 200 nM (35,

48 and 65%, respectively) (Fig. 2b). Therefore, our observations indicated that the increased number of autophagosomes, measured by GFP-LC3 fluorescence, was dose- and time-dependent upon Lipofectamine 2000/NC siRNA 1 complex treatment.

Induction of Autophagosomes by Lipofectamine 2000/NC siRNA Complexes

Confocal microscopy was used to correlate the degree of GFP-LC3 fluorescence with the presence of autophagosomes in Huh7.5 cells. Cells treated with 40 mM glucosamine exhibited an increased number of green fluorescent puncta compared with non-treated cells (Fig. 3). These GFP-LC3 puncta were autophagosomes [11]. A greater number of GFP-LC3 puncta were also observed in Huh7.5 cells treated with 100 nM Lipofectamine 2000/NC siRNA 1 complexes containing 5 µg/ml Lipofectamine 2000, but not in cells treated with 100 nM NC siRNA 1 or with 5 µg/ml Lipofectamine 2000 alone (Fig. 3). These results were similar to those obtained from the quantification of the GFP-LC3 fluorescence (Fig. 2b).

Comparison of Various Negative Control siRNAs

A NC siRNA was originally selected to avoid the possibility that silencing a certain gene would lead to inadvertent autophagy activation. However, to rule out the possibility that NC siRNA 1 had non-specific effects that unintentionally altered cellular autophagy levels, two alternative non-targeting NC siRNAs (NC siRNA 2 and 3) were also evaluated. All of the NC siRNAs 1-3 used in these assays were marketed as having no sequence homology with human genes.

The anti-LC3 Western blot exhibited a slight increase (1.2, 1.5 and 1.2-fold) in the amount of LC3-II in cells treated with 100 nM NC siRNA 1, 2 or 3, respectively (Fig. 4, Lanes 3, 5 and 7). An even greater increase was observed in cells treated with Lipofectamine 2000 complexed with 100 nM NC siRNA 1, 2 or 3 at 24 h (1.6, 2, and 1.7-fold, respectively) (Fig. 4, Lanes 4, 6, 8). Similarly, all three Lipofectamine 2000/NC siRNA complexes at 100 nM elicited comparable and significant induction of GFP-LC3 fluorescence at 72 h (Fig. 5). However, the degree of GFP-LC3 expression by NC siRNA 1 complex was notably higher than those of NC siRNA 2 and 3 complexes (Fig. 5). Thus, using Lipofectamine 2000 to transport different NC siRNAs led to increased relative levels of LC3-II at 24 h and GFP-LC3 fluorescence at 72 h for all of the NC siRNAs tested. This result suggested that any siRNA complexed with Lipofectamine 2000 would lead to a higher number of autophagosomes.

Discussion

In the present study, we demonstrated that the delivery of NC siRNA using Lipofectamine 2000, a commonly used cationic lipid transfection reagent, increased the number of autophagosomes in Huh7.5 cells in a dose- and time-dependent manner. Three independent assays verified these results. The first observation regarded the relative amounts of LC3-II, a widely accepted marker of autophagosome formation [12]. The second observation was based on a novel method of quantifying total cellular GFP-LC3 fluorescence. Finally, confocal microscopy was used to visualize the autophagosomes consisting of GFP-LC3 [14, 21]. We propose that the increased presence of autophagosomes may be an important

response step for the intracellular processing of Lipofectamine 2000/siRNA complexes. Delivery of siRNA is commonly used for genomic mapping [3] and drug target discovery [5]. Thus, it will be of great importance to consider the effects of these complexes during the application. The inadvertent changes to the basal autophagic condition of the cells and subsequently, the overall cellular homeostasis may result in inaccurate observations and conclusions.

Our Western blot results illustrated the conversion of LC3-I to LC3-II. Comparison of LC3-II levels between samples had been reported as the most direct measurement of autophagy induction [12, 14, 21]. Accordingly, higher LC3-II levels correlated with increased Lipofectamine 2000/NC siRNA 1 concentration (Fig. 1b, Lanes 4–6). This observation suggested that the complex led to an increase of autophagosomes in a dose-dependent manner. In addition, Lipofectamine 2000 alone also exhibited higher LC3-II levels (Fig. 1b, Lane 2) compared to the non-treated control, which agreed with a recently published report about autophagy induction by cationic lipids [22]. However, the treatment with 200 nM Lipofectamine 2000/NC siRNA 1 complex resulted in a higher level of LC3-II than the treatment with the same amount of Lipofectamine 2000 or NC siRNA alone (Fig. 1b, Lane 6 vs. Lanes 2 and 3, respectively). Interestingly, 10 µg/ml Lipofectamine 2000 alone did not lead to increased GFP-LC3 compared to control at any time point (Fig. 2b), while the same treatment exhibited LC3 conversion after 48 h treatment (Fig. 1b, Lane 2). The differing responses from these two assays may be due to the difference in the sensitivity of these two different assays.

Similarly, the increase of GFP-LC3 levels was also dependent on the dosage of Lipofectamine 2000/NC siRNA 1 concentration. These observations indicated that the kinetics of GFP-LC3 induction correlated with the concentration of Lipofectamine 2000/NC siRNA 1 complex. Although each concentration of complex treatment produced different amounts of GFP-LC3, they shared a similar trend where the fluorescence levels steadily rose with each time point (Fig. 2b). This dose- and time-dependent pattern suggested that the intracellular processing of internalized Lipofectamine 2000/NC siRNA 1 complexes may promote the GFP-LC3 levels due to the intracellular accumulation of the complexes over time. Even the lowest concentration studied (50 nM) eventually resulted in significantly greater fluorescence levels than no treatment (Fig. 2b). Observations using confocal microscopy further verified that measurement of relative GFP-LC3 levels corresponded with the presence of autophagosomes in Huh7.5 cells (Fig. 3). Therefore, these results suggested that the long-term effects of siRNA complex uptake should be considered because cellular responses may not be immediately apparent.

In order to eliminate the prospect of unintended non-specific effects by NC siRNA 1, two alternate NC siRNAs, NC siRNA 2 and 3, were also used to confirm that the Lipofectamine 2000/NC siRNA complex caused the observed increases of LC3-II and fluorescence. All three NC siRNAs, two from Ambion and one from Qiagen, showed similar results, where the complexes led to greater levels of LC3-II and GFP-LC3, albeit to different extents (Fig. 4, 5). The distinct levels in the results may be due to the unique complexation structures formed by the different siRNA nucleotide sequences and Lipofectamine 2000. Therefore, treatment of various NC siRNAs using Lipofectamine 2000 confirmed two important

conclusions. First, the siRNA sequence influences the degree of autophagy induction. Secondly, and more importantly, any siRNA mixed with Lipofectamine 2000 will affect basal autophagy conditions.

Either autophagy induction or inhibition could account for the observed increase of LC3-II conversion, GFP-LC3 fluorescence and autophagosome presence after the complex treatment [12, 14]. In the former scenario, activation of autophagy requires the formation of autophagosomes, which in turn involves GFP-LC3 production and leads to the conversion of LC3-I to LC3-II. On the other hand, inhibition of autophagy may cause an accumulation of autophagosomes due to decreased autophagic degradation and maturation, which would also result in higher LC3-II and GFP-LC3 levels. We believe the observed increase of LC3-II and GFP-LC3 levels and autophagosomes represent autophagy induction, rather than inhibition, due to the size of the Lipofectamine 2000/siRNA complexes. Uptake of exogenous particles has been reported to generate cellular autophagy [23, 24], and the degree of autophagy induction depended on the size of the particle [25]. Accordingly, siRNA and Lipofectamine 2000 that are mixed to form particles for transfections might be recognized for intracellular processing via the autophagy pathway. Moreover, the proposed handling of particles may be due to the cellular clearance of protein aggregates, one of the primary functions of autophagy [26–28]. Alternatively, the increase in autophagosomes could be due to the cellular response to an increased amount of siRNA delivered intracellularly by Lipofectamine 2000. In this case, one could expect similar results using other siRNA transfection agents.

To our knowledge, this current study is the first report indicating the increased amount of autophagosomes due to siRNA treatment using Lipofectamine 2000. Using several different methods, we observed that Lipofectamine 2000-mediated delivery of siRNA increased the level of autophagosomes in Huh7.5 hepatoma cells. Therefore, it is important to be alert of these effects when Lipofectamine 2000/siRNA complexes are used for transfections. Our studies showed that the effect of the complexes on autophagy increased with prolonged incubation time, where a maximal effect occurred at the latest timepoint tested (72 h). However, the majority of studies in autophagy focus on short-term treatment of 24 h or less. Since siRNA persists intracellularly for ~ 66 h [29, 30], the long-term effects need to be considered, especially in regard to the future development of siRNA delivery systems as therapeutic agents.

Abbreviations

RNAi	RNA interference
siRNA	small interfering RNA
mRNA	messenger RNA
NC siRNA	negative control small interfering RNA
GFP	green fluorescent protein
LC3	microtubule-associated protein 1, light chain 3

DMEM	Dulbecco's modified Eagle's medium
PMSF	phenylmethylsulfonyl fluoride
BCA	Bicinchoninic Acid
RFU	relative fluorescence units
DAPI	4', 6-diamidino-2-phenylindole
PE	phosphotidylethanolamine

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Fig. 1. Western blot of LC3 conversion

(a) Huh7.5 cells were treated with 10 μ M MG-132. Lane 1: OptiMEM. Lane 2: 10 μ M MG-132. (b) Huh7.5 cells were treated for 6 h and then treatment medium was replaced with complete medium for an additional 42 h. Cells were washed with PBS and lysates were collected, run on 15% SDS-PAGE and immunoblotted with anti-LC3 and anti-actin. Lane 1: OptiMEM. Lane 2: 10 μ g/ml Lipofectamine 2000. Lane 3: 200 nM NC siRNA 1. Lane 4: 50

nM Lipofectamine 2000/NC siRNA 1 complex. Lane 5: 100 nM Lipofectamine 2000/NC siRNA 1 complex. Lane 6: 200 nM Lipofectamine 2000/NC siRNA 1 complex.

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Fig. 2. Quantification of GFP-LC3

(a) Huh7.5 cells were treated with 10 µM MG-132 for 24 hours and 40 mM glucosamine for 48 hours. (b) Huh7.5 cells were treated for 6 h with OptiMEM, 10 µg/ml Lipofectamine 2000, 200 nM NC siRNA 1, and 50, 100 and 200 nM Lipofectamine 2000/NC siRNA 1 complexes before being replaced with complete medium. At designated times of 6 h, 24 h, 48 h and 72 h after the start of treatment, cells were washed and lysates were collected. Cell lysate fluorescence was measured at Ex. 485 nm and Em. 518 nm and normalized with protein concentrations quantified using BCA Assay. Data are presented as a percentage of non-treated groups at each time point. Error bars represent standard deviation (n=3). * indicates $p < 0.05$ based on Student's *t*-test for comparison of non-treated group.

Fig. 3. Visualization of autophagosome formation

Huh7.5 cells were treated for 6 h before being replaced with complete medium (except for 40 mM glucosamine where treatment lasted for 48 h). At 48 h from the start of treatment, cells were washed and fixed using 4% para-formaldehyde. Fixed cells' nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and then mounted on slides for confocal microscopy viewing. (Green, GFP-LC3 signal and blue, DAPI signal.)

Fig. 4. Comparison of different NC siRNAs for LC3 conversion

Huh7.5 cells were treated for 6 h before being replaced with complete medium. At 24 h from the start of treatment, cells were washed with PBS and lysates were collected. Lysates were run on 15% SDS-PAGE and immunoblotted with anti-LC3 and anti-actin. Lane 1: OptiMEM. Lane 2: 10 µg/ml Lipofectamine 2000. Lane 3: 100 nM NC siRNA 1. Lane 4: 100 nM Lipofectamine 2000/NC siRNA 1. Lane 5: 100 nM NC siRNA 2. Lane 6: 100 nM Lipofectamine 2000/NC siRNA 2. Lane 7: 100 nM NC siRNA 3. Lane 8: 100 nM Lipofectamine 2000/NC siRNA 3.

Fig. 5. Comparison of different NC siRNAs for GFP-LC3 production

Huh7.5 cells were treated for 6 h before being replaced with complete medium. At 72 h from the start of treatment, cells were washed and lysates were collected. Cell lysate fluorescence was measured at Ex. 485 nm and Em. 518 nm and normalized with protein concentrations quantified using BCA Assay. Data are presented as a percentage of non-treated groups at each time point. Error bars represent standard deviation (n=3). * indicates $p < 0.05$ based on Student's *t*-test for comparison of non-treated group.