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The Efficiency of CD40 Down Regulation by siRNA and Antisense ODN: Comparison of Lipofectamine and FuGENE6

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ABSTRACT

Background: Dendritic cells (DCs) are ideal accessory cells in the field of gene therapy. Delivery of DNA and siRNA into mammalian cells is a useful technique in treating various diseases caused by single gene defects. Selective gene silencing by small interfering RNAs (siRNAs) and antisense oligodeoxynucleotides (ODN)s is an efficient method for the manipulation of cellular functions. An efficient, functional delivery system with no toxicity problems would be attractive. **Objective**: We compared two commercially available cationic lipids, Lipofectamine and FuGENE6, in the delivery of both siRNA and antisense ODNs into mice spleen-derived DCs. **Methods**: Cellular uptake was measured by the means of fluorescein-labelled non-silencing siRNA and antisense ODNs as a model system using flow cytometry. Cytotoxicity of the two delivery systems was compared with propidium iodide and annexin-V staining, and quantified with flow cytometry. The efficiency of our oligonucleotide delivery systems was compared by measuring CD40 expression by flow cytometry. **Results:** CD40 expression in DCs was 38%. After siRNA transfection by Lipofectamine, CD40 expression decreased to 13%, and after transfection by FuGENE6, it decreased to 18%. The difference was statistically significant. CD40 down regulation in DCs transfected with the two different antisense sequences by Lipofectamine was 21% and 23%, and down regulation after transfection by FuGENE6 was 19% and 18%, respectively. The differences were not statistically significant. The effects of siRNA and antisense ODNs were specific. **Conclusion**: Lipofectamine was a more potent delivery system in siRNA effect, followed by FuGENE6. There was no significant difference between Lipofectamine and FuGENE6 as a delivery system of antisense ODNs.

Keywords: Dendritic Cells, siRNA, Anti-Sense Oligonucleotides, Lipofectamine 2000, FuGene6

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INTRODUCTION

DCs are the most powerful and impressive antigen presenting cells because immediately upon facing any antigen in the peripheral tissues, they will uptake and process it (1). Uptake of the antigens can cause DCs to differentiate and mature, and to present the antigens to the T cells for activation and immune response against pathogens (2). These changes occur during the migration of DCs to the secondary lymphoid organs, in which the antigen uptake ability of DCs diminishes (3).

CD40 is a member of tumor necrosis factor (TNF) receptor superfamily, which can be expressed as a co stimulatory molecule on numerous cell types such as DCs. Inhibition of CD40 gene expression in DCs is one of the approaches in immunotherapy. Further, there is a great interest in the treatment of autoimmune diseases by blocking CD40 in new therapies at the stage of preclinical and clinical trials (4).

Antisense ODNs have been employed to inhibit the expression of specific genes since late 1970s (5). Oligonucleotides are anionic macromolecules and can not transfer biological cell membranes and are degraded rapidly by nucleases (6). Because of these limitations, researchers have synthesized various chemical modifications of oligonucleotides (7). Such modified ODN possess disadvantages including decreased mRNA hybridization, higher cytotoxicity and increased unspecific effects (8). Thus, the present study focuses on the delivery of unmodified oligonucleotides using two different delivery systems. In general, there is a strong need for the development of certain delivery systems which will protect the antisense ODNs against enzymatic degradation and provide an enhanced transfection into the cytoplasm of the target cells.

Antisense DNA can degrade RNA and block gene transcription by means of RNaseH (9, 10).

RNA interference (RNAi) is the most efficient post-transcriptional gene silencing mechanism, which can strongly silence the target transcripts (11). This activity is involved in the functional genomics and therapeutic applications as an exciting gene silencing strategy (12-14).

In mammalian cells, long double stranded RNAs (more than 30 nucleotides) will lead to the interferon pathway and a non-sequence specific effect can degrade the target transcripts, but the induction of exogenous siRNA (21-22 nucleotides) in mammalian cells can specifically suppress the heterologous and endogenous genes (15,16).

Choosing a target, designing RNA and antisense ODNs and the transfection method are important factors in the efficacy of siRNA and antisense ODN mediated specific cleavage of the targeted mRNA (17).

Introduction of the siRNA and antisense ODNs into eukaryotic cells may be carried out in two ways: viral and nonviral methods. Viral methods are more efficient than nonviral ones, but since they are time consuming and may raise undesired immune and oncogenic responses, nonviral methods are preferable (18, 19).

For non viral gene transfer, different carriers have so far been introduced by research groups. For example, Felgner et al. (21) were the first researchers who introduced the cationic lipids. These transfection reagents are made of two lipid components i.e. a cationic DNA binding group, and a hydrophobic liposome, which are linked together by a linker molecule. The intracellular release should be mediated by the neutral component (20).

In recent years gene therapy has become attractive due to its potential application in the replacement of dysfunctional genes and the treatment of various incurable diseases (22).

To fabricate non-viral delivery systems, a variety of materials have been utilized (23), which have several advantages like safety, ease of preparation, reproducibility, ability to carry large nucleic acid constructs, and stability (24). Today, many commercial products are available, and we used two of the most common agents, Lipofectamine and FuGENE6, which seem to be highly effective for DNA and RNA oligonucleotides transfection.

Because of the crucial role of DCs in tolerance induction and immunotherapy and also considering the important role of CD40 in DC and T cell interaction, in this study, we compared the efficiency of Lipofectamine and FuGENE6 in the delivery of siRNA and antisense ODNs into DCs to down regulate CD40 expression. The efficiency of transfection was determined using flow cytometric analysis. Selection of a good delivery system is one of the most important steps in this process.

MATERIALS AND METHODS

Animals and Cell Lines. Male Balb/c mice (aged 8 to 10 weeks) were obtained from the Pasteur Institute of Iran (Tehran, Iran). All experiments on the mice were approved and done according the protocols of the Ethical committee of Tarbiat Modares University (Tehran, Iran).

The BCL1 cell line (TIB-197), a mouse B-cell line that constitutively expresses high levels of CD40 (25) was obtained from National Cell Bank of the Pasture institute of Iran and used as the positive control.

All the cells were cultured in RPMI medium (Gibco, USA) supplemented with 10% FCS, 3mM L-glutamine, 5 μM 2ME, 100 IU/ml penicillin and 100μg/ml streptomycin (all prepared from Sigma, USA) and 5% CO_2.

Isolation of DCs from Spleen. DCs were isolated from the mice spleen using CD11c (N418) microbeads (Miltenyi Biotech, Germany) according to the instruction for isolation of DCs provided by the manufacturer.

siRNA Design, Synthesis and Transfection. By means of Dharmacon software (www.Dharmacon.com), the siRNA sequence used for the silencing of murine CD40 was designed. Murine genome database (BLASTn, NCBI, USA) was searched to ensure that the sequences would not target another gene script. siRNA was synthesized by MWG Company (Biotech, Germany) according to the manufacturer's instruction (final concentration, 3μM). The non-silencing siRNA was an irrelevant siRNA, which did not react with any of the target genes. DCs were incubated overnight in 5ml Opti-MEM (Gibco, USA) containing 200 IU GM-CSF (BD, USA). The cells were transfected by FuGENE6 (Roche, Germany) and Lipofectamine (Invitrogen, USA) according to the manufacturers' instructions. To evaluate the siRNA uptake, siRNA was conjugated with FAM (5`) by the manufacturer (MWG, Biotech, Germany). To optimize this protocol, the mature DCs were washed and cultured in Opti-MEM media in 24-well plates (Nunc, Denmark) and then transfected with siRNA at various concentrations of 1, 2, 3 and 4μM of siRNA with 3 and 6μl FuGENE6 or 1and 2μl Lipofectamine for 24, 48 and 72 h.

siRNA sequence was: UCAAGAAACCAAAGGAUAA, and Non-silencing siRNA sequence was: ACGCGAUUGACAUUAGAAU.

Antisense ODN Design, Synthesis and Transfection. The antisense sequence used for silencing of murine CD40 was designed, using oligo analyzer software (www.idtdna.com). The BLASTn (NCBI, USA) was utilized to ensure that the sequences

would not target another gene script. The antisense ODN was synthesized by MWG company (Biotech, Germany) and used according to the manufacturer's instructions (final concentration, 6μM). After an overnight incubation of DCs in Opti-MEM media containing 200 IU GM-CSF (BD, USA), the cells were transfected by FuGENE6 (Roche, Germany) and Lipofectamine (Invitrogen, USA) according to the manufacturer's instructions. To optimize this protocol, the mature DCs were washed and cultured in Opti-MEM media in 24-well plates (Nunc, Denmark) and then transfected with 2, 4, 6 and 8 μM antisense ODN with 3 and 6 μl FuGENE6 or 1 and 2μl Lipofectamine for 24, 48 and 72 h. Transfection with FuGENE6 was performed two times and the second time transfection was performed 24 h after the first one, using the same procedure.
Antisense sequence1 was: ACTCTCTTTAC CATCCTCCT, Antisense sequence2 (25) was: CACAGATGACATTAG, Sense sequence1 was: TGAGAGAAATGGTAG-GAGGA, Sense sequence 2 (25), was: GTGTCTACTGTAATC and Non-silencing Antisense sequence was: CAAGCTCCGATTGCAGAAAG.
Flow Cytometrey. The following antibodies were used for cell surface phenotype analysis: PE-conjugated hamster anti-mice CD11c (Clone HL3) and FITC-conjugated rat anti-mice CD40 (Clone 3/23) together with their respective isotype controls (all from BD Bioscience, USA). For the determination of apoptosis/necrosis, the FITC-conjugated Annexin-V and PI staining methods were used as previously described by others (26). After transfection, the cells were collected on day 2 and were analyzed by flow cytometry (Partec, PAS III, Germany).

RESULTS

Efficiency of siRNA and Antisense ODN Uptake by Splenic DCs in the Presence of Transfection Reagents. Transfection by chemical reagents would be an easy method to evaluate the role of different molecules in DC function.
The DCs were isolated and purified from Balb/c mice and their purity was checked by direct flow cytometry (purity >90%).
To examine whether the DCs were able to internalize naked siRNA and antisense ODN or not, they were cultured with FL-siRNA and FL-antisense in the absence of any transfection reagent. Flow cytometry, estimated that only 2% of the DCs contained incorporated siRNA and antisense ODN (Figure 1E).
The results showed that, at the concentration of 3μM siRNA with 3μl of FuGENE6 and 3 μM of siRNA with 2μl of Lipofectamine, 82.1 % and 80.5% of the DCs and 95.1% and 96.5% of the BCL1 cell line were transfected with siRNA, respectively (Figures 1C,1D).
Also at the concentration of 6 μM antisense ODN with 3μl of FuGENE6 and 6μM of antisense ODN with 2μl of Lipofectamine, 80.9% and 77% of DCs and 94.2% and 95.1% of the BCL1 cell line were transfected with antisense ODN, respecctively (Figures 1A, 1B).
The optimum concentrations of siRNA and antisense ODN were 3μM and 6μM for the DCs and BCL1 cell line and the optimum concentrations of FuGENE6 and Lipofectamine were 3μl and 2μl, respectively.
The ratio of transfection reagents to DNA was 2μl Lipofectamine (1mg/ml) or 3μl FuGENE6 (1mg/ml): 4μg DNA and for RNA, it was 2μl Lipofectamine (1mg/ml) or 3μl of FuGENE6 (1mg/ml): 3μM RNA.

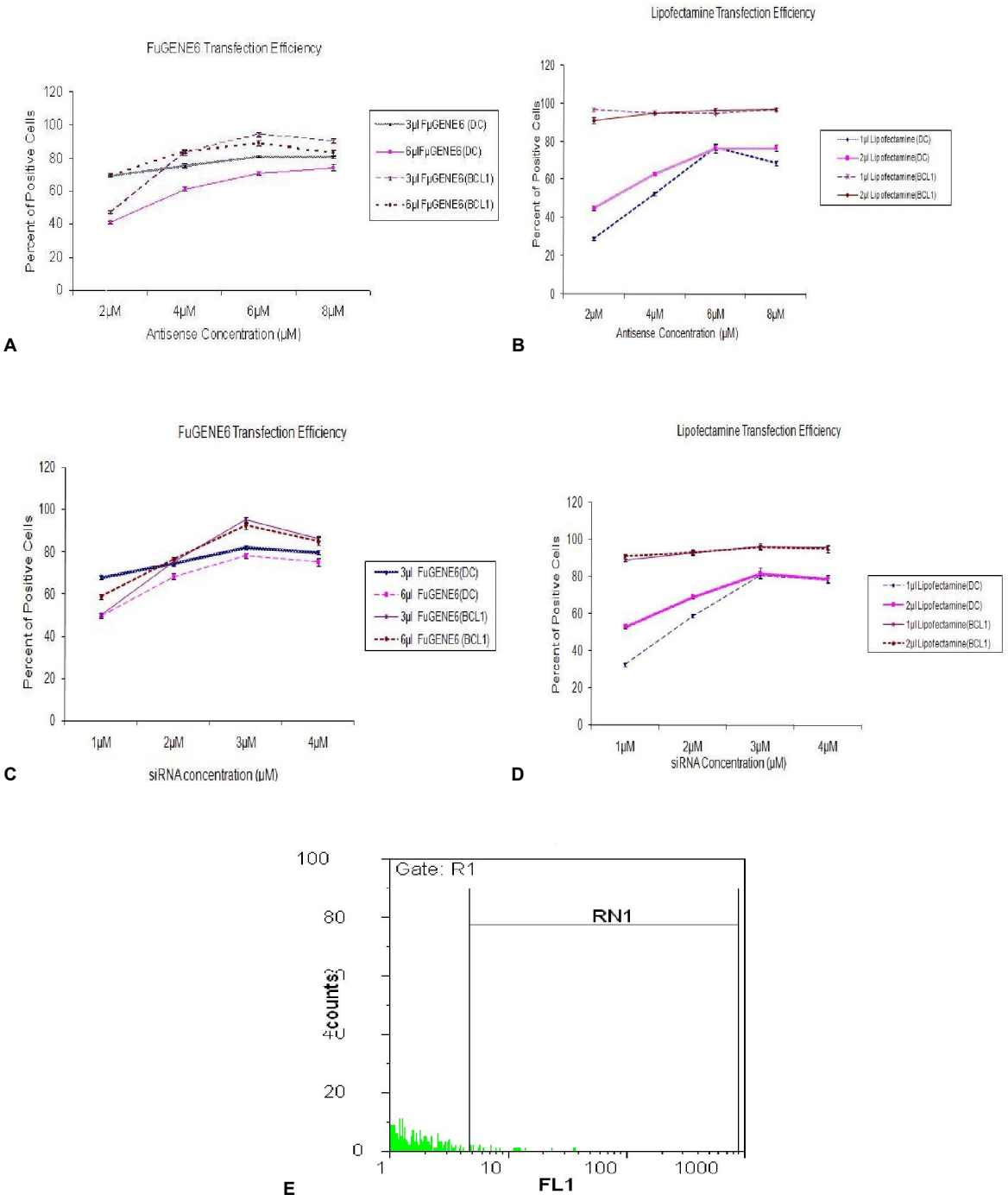

Figure 1. Cell transfection efficiency: The DCs and BCL1 cell line were incubated for 24 h with different floureceine labeled oligonucleotide concentrations in the presence of the transfection reagents. After several washing steps, the transfection efficiency was determined as the percent of fluoreceine positive cells through flow cytometric analysis.
A: FuGENE6 efficiency in antisense ODN transfection, B: Lipofectamine efficiency in antisense ODN transfection, C: FuGENE6 efficiency in siRNA transfection, D: Lipofectamine efficiency in siRNA transfection and E: A representative of our negative control flow cytometric histograms, showing that in the absence of transfection reagents, no labelled siRNA is transferred into DCs. The data are representative of two independent experiments. The colored version is available at: www.iji.ir

Transfection of Splenic DCs with siRNA and Antisense Oligodeoxynucleotide has no Effect on Cell Viability. To understand the probable effect of Lipofectamine and FuGENE6 on the viability of splenic DCs, we stained the splenic DCs with Annexin-V and propidium iodide (PI), and then analyzed them with Partec FlowMax software (Germany).

We observed no toxicity in the transfected cells versus the untreated cells .Therefore, it is concluded that presence of FuGENE6, Lipofectamine together with siRNA and antisense ODN do not affect the viability of transfected DCs in comparison to the untreated cells at the concentrations used. However, Lipofectamine showed cytotoxic effects in higher concentrations (more than 3µl) (Data not shown).

Delivery of CD40 siRNA and Antisense ODN into Splenic DCs Leads to the Down Regulation of CD40 Surface Expression. In order to investigate whether the transfer of siRNA and antisense ODN by chemical reagents (Lipofectamine and FuGENE6) into spleen derived DCs is able to down regulate the CD40 surface expression in DCs we measured the surface expression of CD40 by two color flow cytometry after transfection.

The DCs were transfected with 3µM siRNA or 6µM antisense ODN by either 2µl Lipofectamine or 3µl FuGENE6, and incubated for 24, 48 and 72 h and then stained with respective antibodies (CD11C-PE and CD40-FITC). Finally, they were analyzed by Partec FlowMax software. The optimum time for the decrease in CD40 expression was 48 hours after transfection.

In the BCL1 cell line applying Lipofectamine, the CD40 gene expression was reduced from 69.5% to 43% while the application of FuGENE6 resulted in a decrease in the expression decreased from 69.5% to 46%. These effects were also specific (Figures 2A, 2B, 2C, 2D and 2E).

In the BCL1 cell line, the percentages of CD40 down regulation with two antisense ODNs by means of Lipofectamine were 20% and 18% and by FuGENE6 were 18% and 17%, respectively. The non-silencing strand did not affect CD40 gene expression while the sense strands delivered by Lipofectamine and FuGENE6 reduced the expression by 6% and 8%, respectively (Figures 2F, 2G, 2H, 2I, 2L, 2M and 2N).

The CD40 expression in DCs was 38% and after siRNA transfection by Lipofectamine, it was reduced to 13%. Upon transfection by FuGENE6, CD40 expression was reduced to 18%. The difference was significant ($p=0.042$). The effects of siRNAs were specific because non-silencing siRNAs did not reduce CD40 gene expression (Figures 3A, 3B, 3C, 3D and 3E).

The percentages of CD40 down regulation in DCs transfected with the two different antisense ODNs by Lipofectamine were 21% and 23%, and by FuGENE6 were 19% and 18%, respectively. The differences between these data were not significant ($p=0.052$). The effects of applied antisense ODNs were specific because non-silencing antisense ODN did not affect the down regulation. Sense strand transfections by Lipofectamine and FuGENE6 down regulated the gene expression as much as 3% and 8%, respectively (Figures 3F, 3G, 3H, 3I, 3J, 3K, 3L, 3M, 3N and 3O). To find out if Lipofectamine and FuGENE6 have any effect on CD40 surface gene expression, the expression of CD40 with and without Lipofectamine and FuGENE6 was evaluated by flow cytometry.

The flow cytometry results indicated that Lipofectamine and FuGENE6 did not affect CD40 surface expression in the DCs and BCL1 cell line (Data not shown).

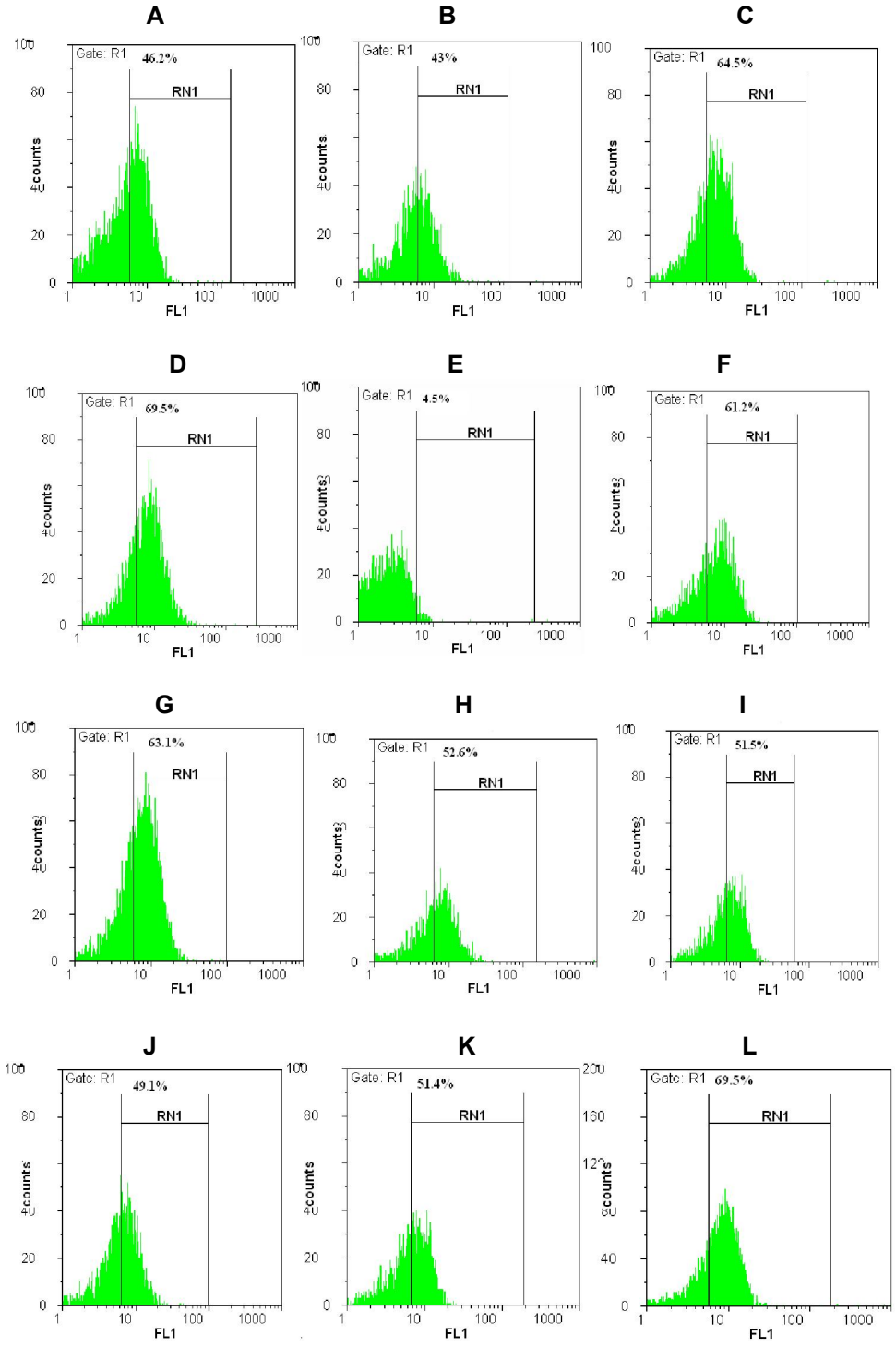

Figure 2. The BCL1 cell line was transfected with CD40 siRNA and antisense ODN using Lipofectamine and FuGENE6 after 48 h. The CD40 expression was measured by flow cytometry. A: CD40 siRNA treated (Lipofectamine), B: CD40 siRNA treated (FuGENE6), C: Non-silencing siRNA (Lipofectamine), D: BCL1 cell line untreated, E: Isotype control, F: CD40 Sense treated (FuGENE6) G: CD40 Sense treated (Lipofectamine), H: CD40 antisense ODN 2 treated (FuGENE6), I: CD40 antisense ODN 1 treated (FuGENE6), J: CD40 antisense ODN1 treated (Lipofectamine), K: CD40 antisense ODN2 treated (Lipofectamine) and L: Non-silencing antisense ODN (Lipofectamine). The data are representative of two independently performed experiments. The colored version is available at: www.iji.ir

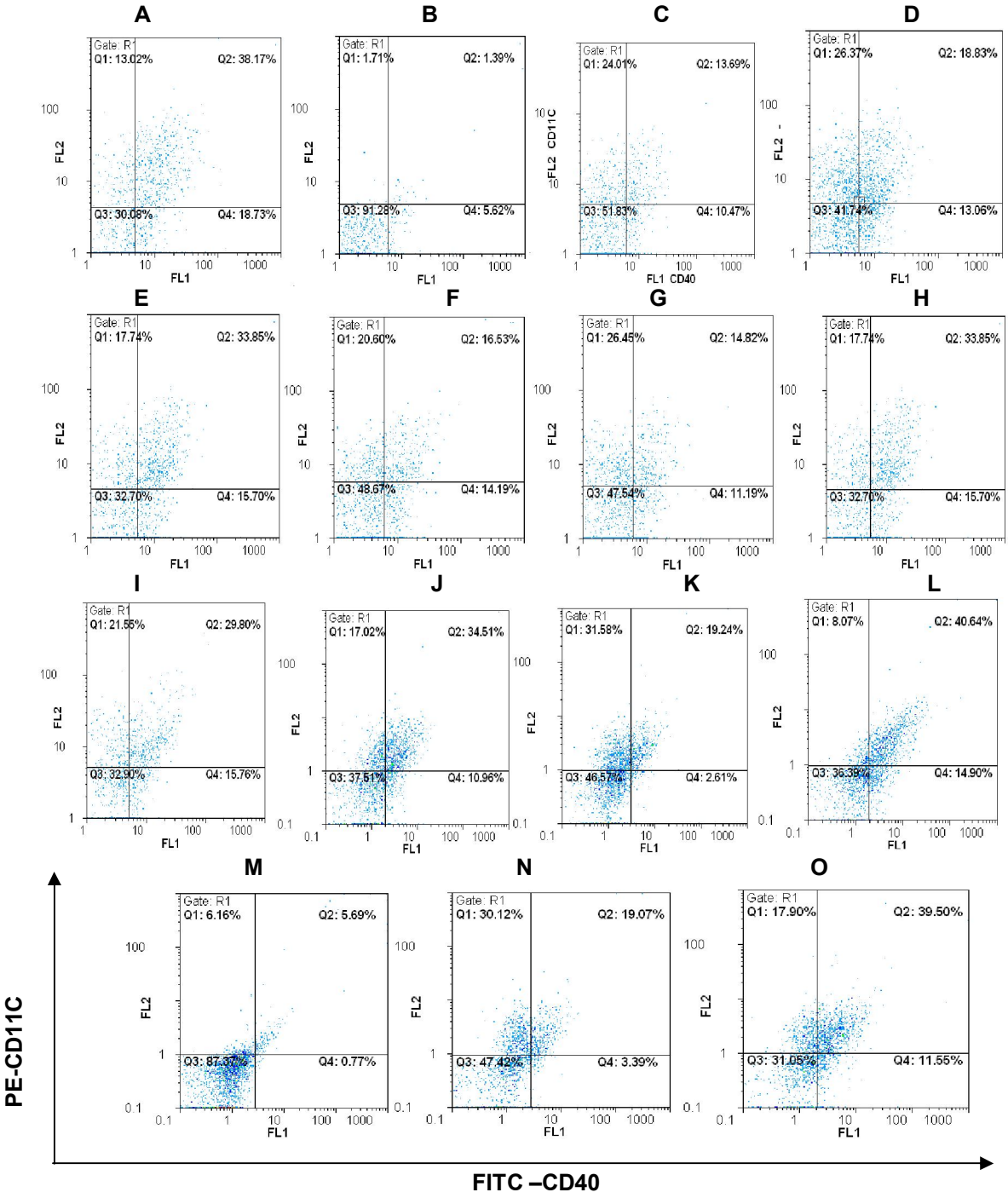

Figure 3. The DCs were transfected by CD40 siRNA and antisense ODN using Lipofectamine and FuGENE6. After 48 h. DC phenotype was assessed by expression analysis of CD40, and CD11c using two color flow cytometry. A: Untreated DC, B: Isotype control, C:CD40 siRNA treated (Lipofectamine), D: CD40 siRNA treated (FuGENE6) E: Non Silencing siRNA (Lipofectamine), F: CD40 antisense ODN1 treated (Lipofectamine), G: CD40 antisense ODN2 treated (Lipofectamine), H: Non Silencing antisense ODN (Lipofectamine), I: CD40 Sense treated (Lipofectamine), J: CD40 Sense treated (FuGENE6), K: CD40 antisense ODN1 treated (FuGENE6), L: Untreated DCs, M: isotype control, N: CD40 antisense ODN2 treated (FuGENE6) and O: Non-Silencing antisense ODN (FuGENE6).
The data are representative of two independently performed experiments. The coloured version is available at: www.iji.ir

DISCUSSION

Gene manipulation of DCs has a role in autoimmune and allograft rejection treatment that is why it has become an exciting topic for many researchers (27). Among the different ways of gene manipulation, the use of the viral transduction methods seems to be effective but is thought to be very dangerous (28). Insertion of the viral genome into the cells can cause oncogenesis or mutagenesis. Also viral proteins can undergo high immunogenicity in the virus manipulated DCs. Accordingly, nonviral methods are now preferable (29).

DCs have important role in immunotherapy and the selection of proper transfection reagent is one of the most important steps in this regard. In this research, we compared the efficiency of two cationic lipids (FuGENE6 and Lipofectamine) for DC and BCL1 cell line transfection by means of siRNA and antisense oligodeoxynucleotides.

Yadava et al. (30) compared two delivery systems for siRNA transfection. They showed that, liposomes in comparison with polyethylenimine (PEI) were better delivery systems for siRNA (30). At the same time, Swami examined a number of delivery systems for DNA/siRNA transfection into mammalian cells and compared the efficiency and cytotoxicity of these systems compared to Lipofectamine and FuGENE6. Swami proposed PEI derivative to be a good delivery system for DNA/siRNA (31).

Until now, there has not been any report for spleen derived DC transfection with Lipofectamine or FuGENE6 as transfectants. Thus, we have to examine various concentrations of Lipofectamine or FuGENE6 to find the optimum amounts of these reagents for DC transfection.

Our flow cytometry results showed that, the efficiencies of cellular uptake of siRNA in DCs by 2µl Lipofectamine and 3µl FuGENE6 were 80% and 82%, respectively.

Also, 80.9% of DCs were transfected with 6µM antisense ODNs by 3µl of FuGENE6, 77% of DCs delivered 6µM of antisense ODN by means of 2µl Lipofectamine.

The percentage of CD40 expression in DCs was 38%. The DCs transfected with siRNA by Lipofectamine and FuGENE6 decreased CD40 expression to 13% and 18%, respectively. Also The DCs transfected with antisense ODNs by Lipofectamine and FuGENE6 decreased CD40 expression to 21%, 23%, and 19%, 18%, respectively.

The differences between FuGENE6 and Lipofectamine in antisense ODN transfection were not significant but in siRNA transfection, there were significant differences between these two delivery systems. It is demonstrated that Lipofectamine is a better for siRNA than for antisense ODNs.

Parameters which had been investigated in this study included the lipid: nucleic acid ratio of the mixture, cytotoxic effect, time of transfection and dose-response effects. It was demonstrated that more than 2µl of Lipofectamine could be cytotoxic in splenic DCs; therefore, a concentration of 2µl of Lipofectamine was used in other work. In other studies, depending upon the cell type used, the concentrations varied (32-33).

It is known that FuGENE6 has very low cytotoxic effect on DCs, while in comparison with other cationic lipid transfection reagents, Lipofectamine is more cytotoxic. It had no effect on DC viability at the concentrations used in agreement with the data of Miller (34). Xiaoyan et al. (35) utilized 3µl of Lipofectamine for antisense ODN transfection of mice bone marrow derived DCs. The differences in cytotoxic effect of this reagent on spleen and bone marrow derived DCs should be further studied. However, Inoue and his co-workers used 4µl of this transfection reagent for several cell line transfections (32).

Nadali et al. (36) used 3µl FuGENE6 for the transfection of K562 (Erythroleukemia) cell line (36). Tao et al. (37) used this transfection reagent for Hela cell line, also Kato et al. (38) used FuGENE6 with a concentration of 3µl/ml for transfection of CHO cell line and showed that the effect is dose dependent and cytotoxicity is very low.
Cell-type and lipid-dependent diversity has been observed in the uptake of oligonucleotide-lipid complexes (39). This fact may be related to a variety of endocytosis cell-dependent rates, and to variable interactions of the positively charged cationic lipid/DNA complexes with anionic residues on the cell surface (40).
It is important to mention that, only one transfection by Lipofectamine is enough to down regulate gene expression, but for FuGENE6, we had to transfect the cells twice. 24 h after the first transfection, the second one can achieve the desired results.
In the last two decades many different delivery systems for oligonucleotides have been developed and now we can demonstrate that the cell delivery is increased by the use of such delivery systems. Also the different delivery systems show different cytotoxic side effects. In conclusion, Lipofectamine in comparison to FuGENE6 is more potent and is a better and more efficient compound in transferring siRNA to splenic dendritic cells, but there is no difference in the efficiencies of Lipofectamine and FuGENE6 in transferring antisense ODNs to splenic dendritic cells and also no differences in their effects on CD40.

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