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Molecules and Cells

Leukemia-Specific siRNA Delivery by Immunonanoplexes Consisting of Anti-JL1 Minibody Conjugated to Oligo-9 Arg-Peptides

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Targeted mRNA degradation by short interfering RNAs (siRNAs) offers a great potential to treat cancers. siRNA therapeutics for leukemias are, however, hindered by poor intracellular uptake, limited blood stability and non-specific delivery. To solve these problems, we developed an anti-JL1 immunonanoplex (antibody-coupled nanocomplex) for siRNA delivery using anti-JL1 minibody (leukemia cell-specific minibody) conjugated to oligo-9-Arg peptide (9R) for effective siRNA delivery to leukemic cells. The anti-JL1 immunonanoplexes were able to deliver siRNA specifically to leukemic cells (CEM and Jurkat), but not to control cancer cells (H9). According to FACS and confocal microscopic analysis, siRNAs delivered by immunonanoplex particles were rapidly taken up by the JL1-positive cancer cells in 2 h. Furthermore, we showed that the anti-JL1 immunonanoplexes were effectively targeted to JL1-positive cells (CEM) inoculated in the mouse bone marrow. These results suggest that the anti-JL1 immunonanoplex is a powerful siRNA delivery system for human leukemia therapies.

INTRODUCTION

RNA interference (RNAi) technology has brought a new modality of treatments for various diseases including genetic diseases, viral diseases, and cancers (Novina and Sharp, 2004). Recently, a number of groups have preclinically tried to treat infectious diseases and cancers by administration of small interfering RNA (siRNA) resulting in reduction of functional gene expression (Song et al., 2003). However, clinical applications of siRNA are hindered by major problems: (1) rapid clearance by the reticuloendothelial system (RES), and enzymatic degradation during circulation and within the cell (Akhtar et al., 2007); (2) poor cellular uptake; (3) non-specific tissue-bioavailability (Chiu et al., 2004; Elmén et al., 2005; Soutschek et al., 2004). To overcome these barriers, various systems for delivery of siRNA have been improved, such as varied viral vectors (Zhou et al., 2009), cationic liposomes (Morrissey et al., 2005), and cationic polymers (Urban-Klein et al., 2005), which have been applied for the delivery of various cargoes into a variety of cells both *in vitro* and *in vivo*. However, most of the approaches were not intended to direct therapeutics specifically to certain types of tissues or cells. The lack of targeted delivery still bears problems of low transfection efficiency as well as nonspecific immune stimulation *in vivo*.

Antibody-mediated targeted delivery would be an effective method for targeting siRNA to a particular type of cell (Pirollo et al., 2008). In this report, we provide evidence that antibody-mediated siRNA delivery is effective in siRNA targeting to T leukemic cells. It is known that hematopoietic cells including T leukemic cells are very reluctant to be transfected by nonviral vectors (Goffinet and Keppler, 2006). For specific siRNA delivery to T leukemic cells, we adopted a mini-antibody (minibody) against JL1 which is a unique antigen of leukemia cells, but not mature hematopoietic cells (Kim and Park, 1998; Park et al., 1998; Shin et al., 2003). Also, we utilized oligo-9-arginine peptides as a cell-penetrating peptide (CPP) for efficient siRNA internalization. CPPs have been shown to significantly improve cellular uptake of various therapeutic molecules both in cultured cells and *in vivo* (Crombez et al., 2007; Ei-Andaloussi et al., 2005). The nine arginine residue conjugated to a neuronal cell-targeting peptide has been also reported to enable siRNA targeting to neuronal cells (Kumar et al., 2007). Accordingly, we have developed a high-performance immunonanoplex delivery system for T-cell leukemia using the anti-JL1 minibody conjugated to the oligo-9-arginine peptide. We proved that the anti-JL1-9Arg conjugate/siRNA complex (anti-JL1 immunonanoplexes) would be an efficient *in vitro/in vivo* siRNA delivery system specific to leukemias.

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MATERIALS AND METHODS

Cell lines and cell culture

Human T leukemic cells, CCRF-CEM (ATCC #CCL-119), Jurkat (ATCC #TIB-152), and H9 (ATCC #CRL-8543), were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Minibody, peptides and mice

Anti-JL1 minibody was prepared by DINONA Inc. (Korea). Briefly, the gene construct of anti-JL1 minibody was made by overlay PCR of scFv gene of anti-JL1 antibody variable region and human Fc region composed of CH2 and CH3 in-frame and then by ligating with pCI-DHFR vector made by in-house. After transfection of the minibody gene construct into CHO-DG44 cells, a stable cell line expressing anti-JL1 minibody was established by limiting dilution and methotrexate (MTX) selection. The minibody was purified by protein-G affinity chromatography from culture supernatants of the established cell line. The purified anti-JL1 minibody was quantified using spectrophotometry at 280 nm. Activity and affinity of the minibody was verified by FACS analysis using a JL1-positive cell line, CEM.

FITC-labeled nonsence siRNAs and oligo-9-arginine (R) peptides were purchased from Bioneer (Korea) and Peptron Inc. (Korea), respectively. NOD/SCID mice were from Korea Research Institute of Bioscience and Biotechnology (Korea).

Preparation of anti-JL1 immunonanoplex

Conjugates of anti-JL1 minibody and 9-arginine peptide were prepared according to a previous report (Kumar et al., 2008) with minor modifications. An anti-JL1 minibody was conjugated a Cys residue at the C-terminal end of 9-arginine peptide, Cys-[Npys]-9R peptide, in 0.1 M phosphate buffer (pH 5.5) (1:10 molar ratio of minibody and peptide) for 4 h at room temperature. Unconjugated 9-arginine peptides were removed by dialysis with a membrane with a MWCO of 10,000 for 48 h. Anti-JL1 immunonanoplexes were freshly prepared by incubating the mixture of anti-JL1-9R conjugates and siRNA (21-mer) for 15 min at room temperature just before utilization.

Gel retardation test

To verify the integrity of anti-JL1-9R/siRNA complexes, 200 pmole of siRNA was incubated with variable amounts of anti-JL1-9R conjugate for 15 min at room temperature. The reaction mixtures were run on 2% agarose gel and uncomplexed free siRNA bands were visualized by UV illumination.

Specific cellular binding of anti-JL1 minibody

In vitro specific immune reactivity of anti-JL1 minibody was evaluated in T-cell leukemia cell lines. CEM (a JL1-positive cell line) and H9 (a JL1-negative cell line) (Shin et al., 2003) were seeded in separate tubes (1×10^6 cells/tube) and treated with fluorescein isothiocyanate (FITC)-labeled anti-JL1 minibody at 4°C for varied periods of time. The cells were washed twice with PBS and were then analyzed by fluorescence-activated cell sorting (FACS) (Beckton Dickinson, USA).

In vitro transfection by anti-JL1 immunonanoplexes

For *in vitro* transfection with the anti-JL1 immunonanoplexes, JL1-overexpressing CEM cells and JL1-low-expressing Jurkat cells were prepared in 5 ml tubes cells (1×10^6 cells per tube). FITC-siRNA (200 pmole) complexed with anti-JL1-9R conjugates at varied molar ratios were added to the prepared cells. After varied time of incubation at 37°C, the cells were washed twice with PBS (pH 7.4) and analyzed by FACS and confocal microscopy (Fluoview-FV300, Olympus, Japan).

In vivo transfection by anti-JL1 immunonanoplexes in a CEM leukemia mouse model

Mice carrying CEM leukemia were prepared as described previously (Kushida et al., 2001). In brief, pre-patella skin of 7-week-old female NOD-SCID mice was incised in 1 cm length under anesthesia. CEM (1×10^7) cells in 30 μl of PBS were directly injected into the joint surface of tibia through patella tendon and then inserted into the bone marrow cavity. The next day, 1 nmole of FITC-conjugated siRNA which were complexed with unconjugated 9R peptides or anti-JL1-9R conjugates (1:8 molar ratio of siRNA and 9R, respectively) was injected into the bone marrow of SCID mouse bearing CEM leukemia. At 2 h post injection, harvested bone marrow cells were stained with PE-conjugated anti human MHC class I antibody for 30 min at 4°C. The live cells were gated as the propidium iodide-negative population and analyzed using a FACSCalibur flow cytometer (Beckton-Dickinson, USA) and CellQuest Pro software.

RESULTS

Specific cellular binding of anti-JL1 minibody

To evaluate specific immune reactivity of anti-JL1 minibody, *in vitro* cellular binding assay was performed with H9 and CEM cells using FITC-labeled JL1 minibodies (Fig. 1A). CEM and H9 cells were incubated in the presence of FITC-labeled JL1 minibodies for 15 min to 3 h and binding of the FITC-labeled JL1 minibodies to the cell surface were analyzed by FACS. JL1 minibodies were able to effectively bind to the surface of CEM cells over-expressing JL1 antigens while rarely bind to the negative control H9 cells not expressing JL1.

Formation of stable immunonanoplex consisting of anti-JL1-9R and siRNA

Anti-JL1-9R conjugates were complexed with FITC-siRNA at varied ratios and then loaded in agarose gel to examine their stable complex formation. As shown in Fig. 1B, formation of immunonanoplexes was initiated at 1:1 molar ratio of anti-JL1-9R and siRNA. No free siRNA was detected at the molar ratio of 6:1, implying siRNA molecules are entirely associated with anti-JL1-9R conjugates.

In vitro leukemic cell-specific siRNA delivery mediated by anti-JL1 immunonanoplexes

Next, the siRNA-transferring potency of JL1 immunonanoplexes was tested in the prepared human T leukemic cells. The leukemic cells were treated with a fixed amount of FITC-siRNA (200 pmole) complexed with varied amounts of anti-JL1-9R peptide and siRNA transfection was monitored by FACS at varied time points. T-cell specific transfection mediated by the anti-JL1 immunonanoplexes was demonstrated in three cancer cell lines, JL1-negative H9 cells, JL1-over-expressing CEM cells and JL1-low-expressing Jurkat cells. Specific and significant cellular uptake of siRNA was observed in CEM cells (Fig. 2B) compared with the control H9 cells (Fig. 2A). Moreover, the transfection efficiency of the immunonanoplex was directly related to the molar ratio of anti-JL1-9R:siRNA and incubation time. At 8:1 molar ratio, 96% of the treated CEM cells were transfected with FITC-siRNA in 2 h while only 5.7% of H9 cells were transfected under the same conditions. Meanwhile, at 12:1 molar ratio the immunonanoplexes exhibited less efficient transfection to CEM cells and rather higher non-specific transfection to H9 cells. At 3 h after transfection, the percentile of

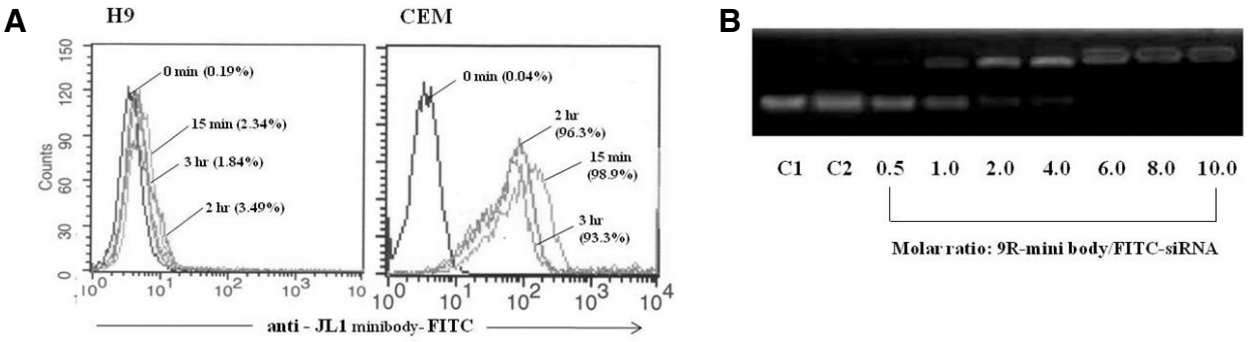


Fig. 1. Immunoreactivity of anti-JL1 minibody and anti-JL1 immunonanoplex formation with siRNA molecules. Human T leukemia H9 (JL1-negative) and CEM (JL1-positive) cells were treated with FITC labeled anti-JL1 minibodies and then analyzed by FACS after incubation for 15 min, 2 h and 3 h (A). Anti-JL1-9R and siRNA were mixed at the indicated molar ratios of anti-JL1-9R and siRNA, incubated for 15 min, and then run on 2% TAE agarose gels (B). The percentile represents the ratio of cell bound to anti-JL1 minibodies.

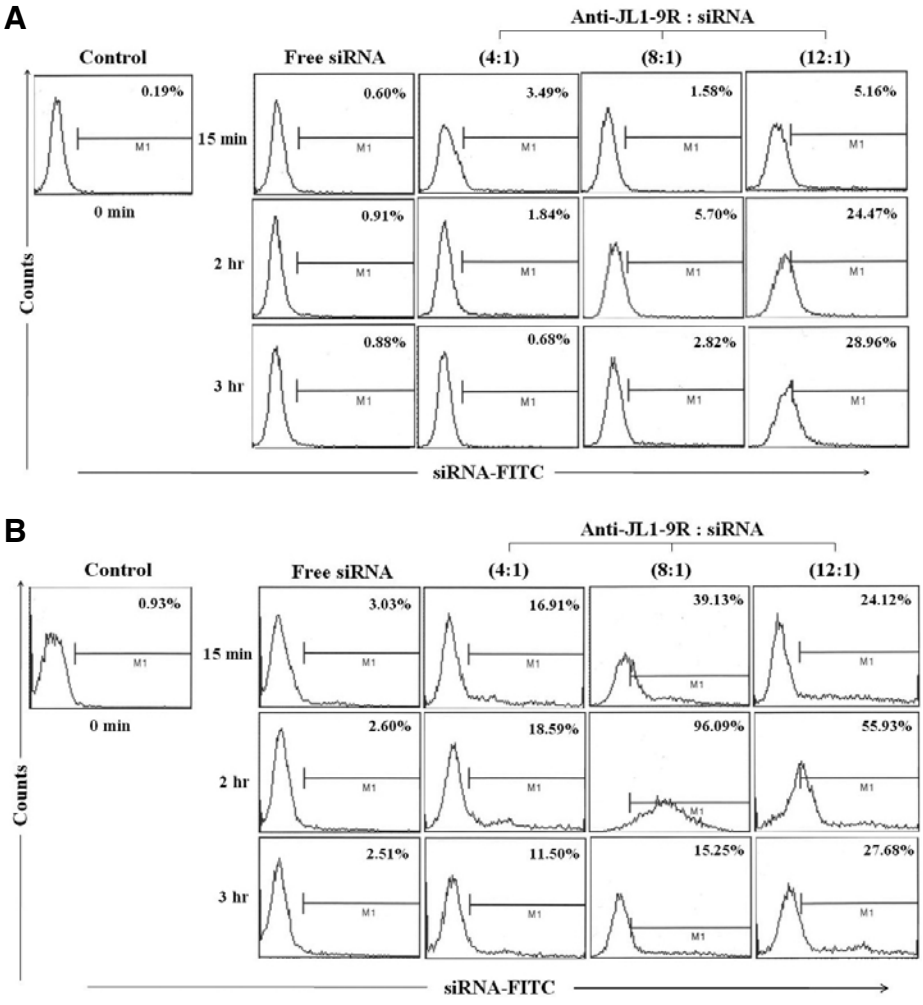
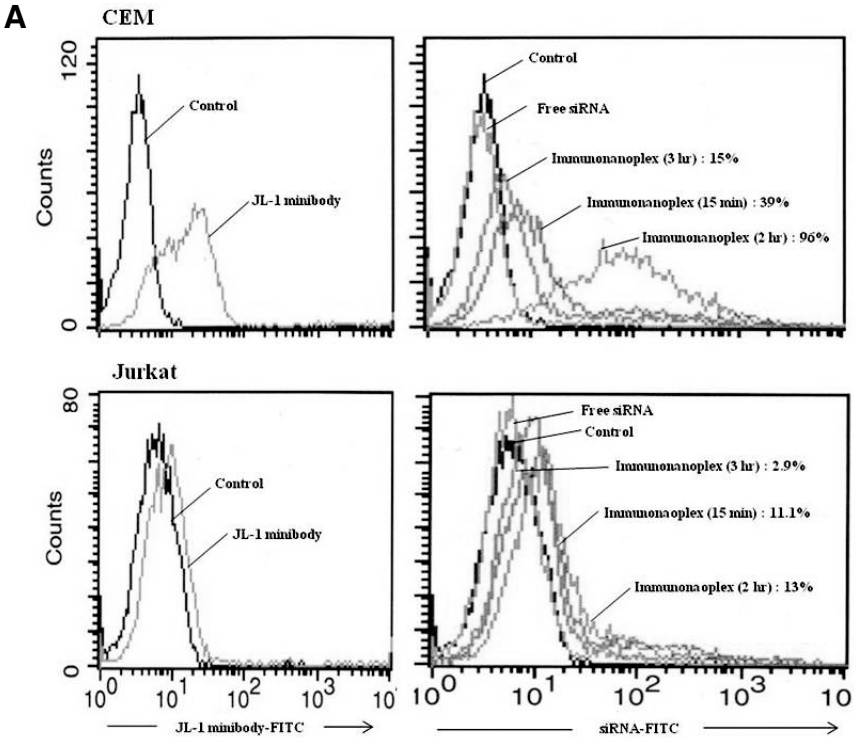


Fig. 2. Effects of the molar ratio of anti-JL1-9R and siRNA on transfection to CEM and H9 cells. H9 (A) and CEM (B) cells were transfected with free siRNA-FITC or anti-JL1-9R: siRNA-FITC immunonanoplexes prepared at varied molar ratios and analyzed by FACS at three different time points. The percentile represents the ratio of siRNA transfected cells.

transfected CEM cells decreased to 15%.

In order to verify JL1-dependent transfection to leukemic cells, cellular binding and transfection efficiency of the anti-JL1 immunonanoplex were examined in CEM and Jurkat cells by FACS and confocal microscopic analysis (Fig. 3). FITC-labeled JL1 minibodies exhibited a higher binding affinity to CEM cells over-expressing JL1 antigens than Jurkat cells low-expressing JL1 (Fig. 3A). Also, according to the confocal microscopic pictures of the transfected cells, CEM cells were more receptive to FITC-siRNA complexed with anti-JL1-9R into the cytoplasm than Jurkat cells (Fig. 3B). These results show that the transfection efficiency of the anti-JL1 immunonanoplexes can be varied depending on the concentration of JL1 molecules expressed on leukemic cell surface.



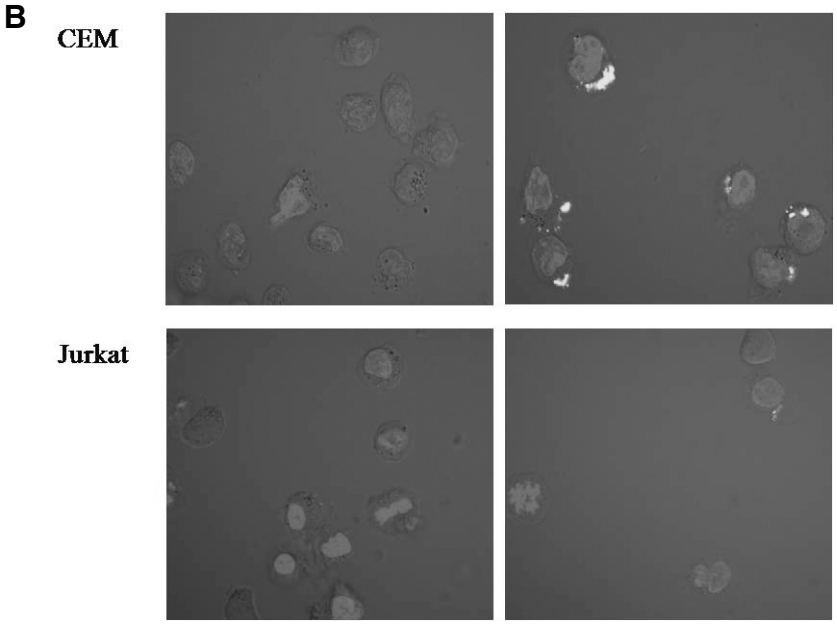


Fig. 3. JL1-dependent siRNA transfection mediated by anti-JL1 immunonanoplexes. Anti-JL1-9R peptides complexed with 200 pmole of FITC-siRNA (8:1 molar ratio) were added to JL1-overexpressing CEM cells and JL1-low-expressing Jurkat cells. The FITC-siRNA-transfected cells were analyzed by FACS (A) at varied time points. The percentile represents the ratio of siRNA transfected cells. The transfected cells were also visualized by confocal microscopy 2 h post transfection with free siRNA or anti-JL1 immunonaoplexes (B).

Anti-JL1-mediated siRNA delivery to leukemic cells in the mouse bone marrow

Anti-JL1-mediated siRNA delivery specific to leukemic cells was examined in a NOD/SCID mouse model carrying human CEM leukemia. JL1-9R:siRNA complexes (8:1 molar ratio) were injected into the bone marrow of leukemic mice. According to the FACS analysis of bone marrow cells (Fig. 4), anti-JL1 immunonanoplexes were able to deliver siRNA-FITC to 7.32% of total CEM cells (3.28% of bone marrow cells) while the control JL1-defficent 9R nanoplexes were little effective. These results also support the leukemic cell-specific siRNA delivery mediated by the anti-JL1 immunonanoplex.

DISCUSSION

To be suitable for target cell-directed siRNA delivery, several strategies have been suggested (Guo et al., 2005; Pirollo et al., 2006; Schiffelers et al., 2004; Vornlocher, 2006; Williams, 2005). One of the promising strategies is antibody-mediated siRNA delivery specific to a certain type of cell or tissue, e.g. T-cells

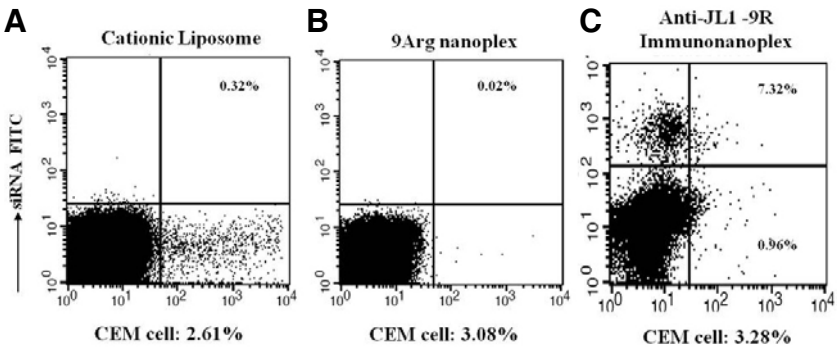


Fig. 4. *In vivo* siRNA delivery to T leukemic cells grown in the mouse bone marrow. One nmole of FITC-siRNA complexed with 9R peptides (B) or anti-JL1-9R peptides (C) at 1:8 molar ratio of siRNA and peptide were administered into the bone marrow of a NOD-SCID mouse bearing CEM leukemia. The transfected bone marrow cells were harvested 2 h post injection and examined by FACS analysis. The percentile represents the ratio of siRNA transfected cells in human MHC class I (hMHC I)-positive (CEM cells) or -negative (mouse bone marrow cells).

(Liu, 2007). In this study we have successfully developed anti-JL1 immunonanoplexes by providing the leukemic cell-specific properties of anti-JL1 minibody to 9-arginine peptides. The anti-JL1-9R peptides were able to be effectively complexed with siRNA at an appropriate molar ratio of peptide and siRNA, resulting formation of stable immunonanoplexes. The delivery of siRNA by the anti-JL1 immunonanoplex is particularly attractive for the following reasons. Firstly, the integrity of siRNA can be stably maintained during blood circulation because the complexation by cationic polyarginine peptides interferes with attacking of digestive enzymes. Secondly, leukemic cell-specific siRNA delivery can enhance its therapeutic efficacy and, therefore, reduce aberrant side-effects by lower dosing of therapeutic siRNA. Lastly, 9-arginine peptides, which are known to be a potent CPP, in the immunonanoplex may enhance siRNA internalization into the target cells after cellular recognition by the coupled antibody molecules. In fact, the anti-JL1 immunonanoplex was able to mediate efficient siRNA delivery specific to leukemic cells *in vitro* as well as *in vivo*. The anti-JL1 immunonanoplex prepared under the optimized conditions (8:1 molar ratio of peptide and siRNA) exhibited efficient siRNA transfer only to CEM cells (96% transfection efficiency), not to H9 cells (5.7%). In addition, the same system also showed leukemic cell-specific siRNA delivery in a mouse leukemia model. The anti-JL1 immunonanoplex was able to deliver to 7.3% of CEM cells, but not to neighboring all types of bone marrow cells. Due to the leukemia-specific JL1 epitopes presented on various types of immature leukemic cells but not on mature peripheral bone marrow cells and bone marrow stem cells (Kim and Park, 1998; Park et al., 1993; 1998; Shin et al., 2001), anti-JL1 minibody-conjugated immunonanoplexs could be a versatile delivery system targeting to most categories of leukemias, especially T cell leukemia shown in this study.

The siRNA-transferring efficiency of anti-JL1 immunonanoplexes was depending on the concentration of JL1 molecules expressed on leukemic cell surface. JL1-overexpressing CEM cells were more receptive to the anti-JL1 immunonanoplexes than JL1-low-expressing Jurkat cells, rendering higher siRNA transfer to CEM cells than Jurkat cells. These results also support that the effective siRNA delivery to T leukemic cells is mediated by the anti-JL1 minibodies conjugated to the nanocomplexes of siRNA. According to the results of time-dependent siRNA transfection, siRNAs delivered by the anti-JL1 immunonanoplexes were rapidly taken up in 2 h and then rapidly degraded inside cells as previously reported (Swami et al., 2007). These results evidenced again that longer surviving chemically modified siRNA structure is mandatory for therapeutic applications of siRNA.

Presumably, the stable nanocomplex formation was mediated by charge interactions between negatively charged siRNA and positively charged poly-arginine peptides as suggested previously (Kumar et al., 2008). According to our study, appropriate electronic interactions are crucial for formation stable and cell-specific anti-JL1-9R/siRNA complexes. Anti-JL1-9R and siRNA began to be complexed at of 4:1 molar ratio of peptide and siRNA. However, those immunonanoplexes were inefficient in siRNA transfection to T leukemic cells. Interestingly, the imminonanoplex at 12:1 molar ratio was not only less efficient in siRNA transfer to CEM cells, but also exhibited rather nonspecific transfection to H9 cells. The nonspecific transfection may be due to remnant cationic polyarginine molecules, not involved in interacting with siRNA, which can provoke nonspecific charge interaction with the cell.

The major limitations of the peptide delivery systems developed so far appear to be their excessive positive charge and lack of target cell specificity, which resulted in non-specific tissue distribution and aberrant immunogenic toxicity (Morris et al., 2001). Target cell-specific siRNA delivery systems including the anti-JL1 immunonanoplex may relieve those problems significantly. Based on the experimental results in this study, anti-JL1 immunonanoplexes can be served as an innovative system for systemic siRNA delivery to leukemic cells. This system may also significantly expand the prospect of siRNA-based therapeutics for various classes of leukemias that apparently express JL1 on their cell surface.

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