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Penetration and efficacy of VEGF siRNA using polyelectrolyte complex micelles in a human solid tumor model *in-vitro*

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A B S T R A C T

A polyelectrolyte complex(PEC) micelle-based siRNA delivery system has been developed for vascular endothelial growth factor (VEGF), and its antitumor efficacy has been demonstrated using *in-vivo* animal models. Penetration and distribution through the avascular regions of human solid tumors after extravasation are important issues for antitumor efficacy, especially for macromolecules such as VEGF siRNA PEC micelles. Using an *in-vitro* solid tumor model, multicellular layers(MCL) culture of human colorectal cancer cells, we evaluated the penetration kinetics and efficacy of VEGF siRNA PEC micelles(PEC-siRNA) in comparison to unmodified siRNA(N-siRNA). The PEC-siRNA showed full penetration (15–17 layers of cells) with a unique punctuated distribution pattern at 48 h following initial accumulation in the top layers and a significant suppression of mRNA and protein expression in a dose-dependent manner after 72 h exposure. Although the initial penetration of N-siRNA was faster than that of PEC-siRNA, N-siRNA showed complete loss of activity due to its instability within 24 h. Our data support the idea that PEC micelle formulation may provide stable penetration tool through the multilayers of cancer cells and ensure the gene silencing effect of VEGF. This study also demonstrated that MCL could serve as a useful in-vitro model to evaluate the dose- and time-dependent profiles of penetration and efficacy of macromolecular delivery systems in human solid tumor avascular regions.

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1. Introduction

RNA interference(RNAi) was first identified in *C. elegans* in 1998 and subsequently in mammalian cells as a post transcriptional gene silencing mechanism [1]. RNAi has developed within a decade into a tool for functional molecular genetics, target gene validation in drug discovery, and a novel therapeutic strategy [2,3]. Ever since evidence first appeared concerning its therapeutic potential in fulminant hepatitis [4], small interfering RNA (siRNA)-induced RNAi has been considered as a treatment modality for human diseases, including cancers [5,6]. Stability in biological fluids and effective delivery constitute the main challenges for siRNA therapeutics [7–9]. Despite the lower transfection efficiency, non-viral delivery systems are preferred due to their safety, low immunogenicity, and relatively low cost and greater reproducibility for large scale production [10]. Several drug delivery systems (DDS) such as, neutral liposomes [11], cationic cardiolipin liposomes [12], cationized gelatin [13], and atelocollagens [14] have been used to improve the stability and intracellular delivery of siRNA. Polyethylenimine (PEI) is the most widely used cationic polymeric gene carriers which and it shows superior transfection efficiency and an endosomal proton sponge effect [10,15,16].

Human malignancies are targets disease for siRNA-based therapeutics, with a long list of potential targets related to aberrant signaling pathways in cancer cells [17]. Angiogenesis in human solid tumors is drawing much attention as a direct treatment target and a basis for universal chemosensitization [18]. Vascular endothelial growth factor (VEGF) is an important angiogenic factor associated with tumor growth and metastasis in a wide variety of solid tumors [19–21]. A delivery system of polyelectrolyte complex (PEC) micelles for VEGF siRNA has been developed using poly ethylene glycol (PEG)-conjugated siRNA and cationic PEI, which show enhanced stability and cellular uptake [22]. The significant anti-tumor effect of these VEGF siRNA PEC micelles also has been demonstrated *in-vivo* in animal models with inhibition of VEGF expression and enhanced tumor accumulation, as previously reported [23].

In solid tumors, preferential accumulation of macromolecules such as siRNA complex may be attributable to enhanced permeability and retention (EPR) effect [24,25]. After extravasation, however, macromolecules or released drug need to penetrate and distribute throughout

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the avascular tumor region to achieve an effective concentration. The avascular distribution/delivery of anticancer agents has drawn a great deal of attention as one of the main determinants of drug efficacy in human solid tumors [26,27]. Large molecules penetrate much more slowly than small compounds do into the inner regions of solid tumors, which may result in compromised activity *in-vivo* [28]. Three dimensional (3D) *in-vitro* models of human solid tumors, including multicellular layers (MCLs), mimic the *in-vivo* condition of solid tumor avascular microregions, including the 3D architecture, cell density, abundance of extracellular matrix (ECM), and cell–cell and cell-ECM communication. These models are the best *in-vitro* models for studying spatial drug distribution as a function of tissue depth (distance from drug exposure side), and possibly, in relation to its activity *in-situ* [29,30]. In a previous study, we successfully used the MCL model to study the penetration and cytotoxicity of paclitaxel [31].

In the present study, we used an MCL culture of human colorectal cancer cells to evaluate the penetration and efficacy of VEGF siRNA formulated as PEC micelles. The penetration over multicellular layers was evaluated using Cy3-labeled PEC micelles and comparison was made between the PEC formulation (PEC-siRNA) and non-formulated, "naked" siRNA (N-siRNA). The efficacy of RNAi and subsequent decrease in VEGF expression were evaluated using *in-situ* hybridization and IHC, respectively.

2. Materials and methods

2.1. Chemicals and reagents

A transwell insert® (0.4 μm microporous membrane) was purchased from Corning Costar (Acton, MA). Methoxy-poly ethylene glycol derivatized with a sulfhydryl group (mPEG-SH, Mw 5000) was obtained from Nektar (Huntsville, AL). *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) was obtained from Pierce (Rockford, IL). Branched polyethylenimine (PEI, Mw 25,000), L-glutathione (GSH), and penicillin/streptomycin solution were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), RPMI-1640 medium, and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (Grand Island, NY). All other chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. PEC micelles formulation and Cy3-labeling of siRNA

VEGF siRNA was purchased from Bioneer Co. (Daejeon, South Korea). The target sequence of the VEGF siRNA was GGAGTACCCTGATGAGATC (human VEGF, bases 189–207); sense strand, 5′-GGAGUACCCUGAUGAGAUCdTdT-3′ and antisense strand, 5′-GAUCUCAUCAGGGUACUCCdTdT-3′. The PEG conjugate, siRNA-s-s-PEG (siRNA-PEG) was prepared as previously described [22,23]. Briefly, the 3′-end hexylamine modified siRNA (Bioneer Co. Daejeon, South Korea) was activated with the disulfide cross linker, *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and then coupled with mPEG-SH (Mw 5000) to produce siRNA-PEG. The conjugation efficiency of siRNA-PEG was about 80%, as determined by HPLC analysis. After HPLC purification, more than 95% purity was achieved for siRNA-PEG conjugates. For dye conjugation, the sense strand of the siRNA was further modified to link a Cy3 group at its 5′-end prior to PEGylation (Dharmacon Research, Lafayette, CO). To prepare siRNA PEC micelles, the siRNA-PEG conjugate was simply mixed with PEI at an N/P ratio of 16 and incubated at room temperature for 15 min [22,23].

2.3. Stability of PEC-siRNA

The stability of unmodified N-siRNA and PEC-siRNA was investigated by incubating each formulation (10 μg siRNA) in DLD-1 MCL conditioned media containing 10% FBS at 37 °C. Aliquots taken at the indicated time intervals were analyzed by gel electrophoresis (TBE buffer, 2% agarose gel). Heparin sodium salt solution (50 mg/mL) was used to decomplex siRNA-PEC by quenching the cationic PEI core and liberating the free siRNA-PEG prior to gel electrophoresis, as previously described [22].

2.4. Culture of multicellular layers (MCLs)

The human colorectal cancer cell line DLD-1 was obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were maintained in RPMI-1640 supplemented with 100 μg/mL streptomycin, 100 units/mL penicillin and 10% heat-inactivated FBS in a humidified, 5% (v/v) CO_2 atmosphere at 37 °C. Cells in log phase were harvested using trypsin and plated on collagen-coated, microporous (0.4 μm) membranes in transwell inserts at a density of 3×10^5 cells/100 μL. The six inserts were submerged in a culture jar supplemented with 150 mL of RPMI-1640 media with continuous stirring in the bottom chamber [31]. After 7 days of culture (MCL thickness: approximately 250 μm), each transwell insert was transferred to a six-well plate containing 7 mL of media/well for subsequent experiments. MCLs were maintained under the same conditions the monolayer cultures were.

2.5. siRNA distribution within the MCL of DLD-1 cells

MCLs were exposed to siRNA by adding Cy3 labeled N-siRNA or PEC-siRNA (200 pmol/200 μl) to the top chamber of the transwell and maintained for up to 72 h. Frozen sections (20 μm) of the MCLs were prepared in vertical direction (perpendicular to the transwell membrane) and a fluorescence microscope (Olympus, AX70, TR-6A02, Tokyo, Japan) was used to obtain fluorescent images of the frozen sections at $\lambda_{Ex/Em} = 482/505$ nm. Line morphometric analysis of fluorescence intensity was performed using Optimas Image Analysis software ver. 6.5 (Media Cybernetics, Buckinghamshire, UK). Data were normalized for tissue auto-fluorescence and plotted against the relative distance (%) from the top surface (drug exposure side) of the MCLs.

2.6. In-situ hybridization and immunohistochemistry for VEGF on MCL

The riboprobe sequence was generated from the rat cDNA (393-bp) for VEGF-A (kindly provided by Dr. Lawrence F. Brown, Harvard Medical School, Boston, MA) and included sequences homologous in all four isoforms of VEGF-A [32]. This sequence was identified to have over 90% homology with the human VEGF-A sequence on a search of the NCBI/BLAST databases. Digoxigenin-labeled antisense and sense riboprobes were prepared as previously described [33]. Specificity and selectivity were confirmed using human adult kidney and developing neuronal tissue samples as negative and positive controls, respectively.

MCLs were exposed to N-siRNA or PEC-siRNA (20 and 200 pmol/200 μl) for 72 h at 37 °C in a CO_2 incubator. At the end of the exposure time, MCLs were cut out of the transwells with membranes attached on, fixed in 4% paraformaldehyde and embedded in paraffin wax. Vertical cross-sections (5 μm) were dewaxed, and treated in 0.2 N HCl for 30 min followed by pepsin (0.02 mg/ml for 15 min) treatment at room temperature. The sections were post-fixed in 4% paraformaldehyde, rinsed in PBS, dehydrated with a graded ethanol series, and dried. Pre-hybridization was performed for 2 h at 53 °C in 50% formamide, 4× SSC, 10% dextran sulfate, 1× Denhardt's solution, and 1 mg/ml salmon sperm DNA. Hybridization was subsequently performed for 15 h in the same solution minus the salmon sperm DNA, which was substituted with VEGF-A antisense riboprobe (400 ng/ml). Sections were washed and incubated with anti-digoxigenin antiserum conjugated with alkaline phosphatase, and histochemical detection was then performed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim GmbH, Mannheim, Germany). All section images were

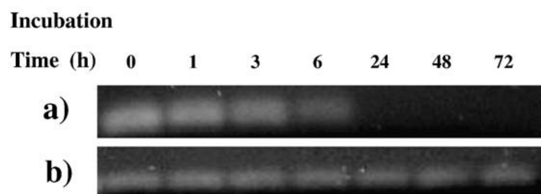

Fig. 1. Stability of PEC-siRNA. Stability of naked siRNA (a) and PEC-siRNA (b) after incubation in conditioned media containing 10% FBS at 37 °C for up to 72 h.

obtained with an Olympus AX70 microscope connected to a CCD Camera using the same light intensity and exposure. The line morphometric analysis of the pixel density was done using saturation parameter (Optimas image analysis software ver 6.5, Media Cybernetics). For negative control, adjacent sections were also processed for *in-situ* hybridization using sense riboprobe instead of an antisense probe, and no staining was observed.

For immunohistochemistry of VEGF protein, 5 μm MCLs sections were obtained for *in-situ* hybridization, as described above. After dewaxing and rehydration, sections were incubated with 3% H_2O_2 for 30 min to eliminate endogenous peroxidase activity. Non-specific binding sites were blocked with normal donkey serum for 30 min and incubated overnight at 4 °C in mouse antiserum against VEGF-A (dilution 1:100; Chemicon International Inc., Temecula, CA). After rinsing in PBS, sections were incubated in peroxidase-conjugated donkey anti-mouse IgG (dilution 1:200; Jackson ImmunoResearch Lab, Inc. West Grove, PA) for 1 h. For coloration, PBS-washed sections were incubated at room temperature with a mixture of 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 until a brown color was visible, and then washed with PBS, counterstained with hematoxylin, and mounted.

2.7. ELISA analysis for VEGF protein in medium

MCLs were exposed to N-siRNA or PEC-siRNA (20, 60 and 200 pmol/200 μl) for 72 h under cell culture conditions. At the end of the exposure time, the culture medium in the bottom chamber was collected and centrifuged to remove cell debris. The amount of VEGF secreted from the MCLs was determined using a Quantikine® human VEGF immunoassay kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

2.8. Statistical analysis

Data are presented as the mean ± SEM. Multiple comparisons were performed using one-way analysis of variance (ANOVA) and the post hoc Turkey test in order to determine in order to determine statistical significance (SPSS® for Windows, version 11.0.0). p values <0.05 were considered statistically significant.

3. Results

3.1. Stability of VEGF siRNA PEC micelles

Anti-VEGF siRNA was conjugated to PEG via a disulfide bond, which is cleavable under intracellular reducing conditions [22]. The PEC micelles were prepared according to the method previously reported [22], and they showed no significant aggregation under current experimental conditions (data not shown).

The stability of both N-siRNA and PEC-siRNA was evaluated in conditioned media of DLD-1 MCLs for 72 h. The N-siRNA degraded rapidly, and no intact siRNA was observed after 24 h, whereas the PEC-siRNA showed no significant changes in siRNA amount until 72 h, indicating superior stability (Fig. 1). The increased stability of the PEC micelle formulation may be attributable to the PEG coronal layer surrounding the PEC structure [22].

3.2. Distribution of anti-VEGF siRNA within MCLs of DLD-1 cells

Distribution of siRNA was evaluated within the MCLs of DLD-1 cells upon exposure to Cy3-labeled N-siRNA or PEC-siRNA (200 pmol). At 6 h post exposure, both the N-siRNA and PEC-siRNA showed distribution limited to the upper layers of the DLD-1 MCL (Fig. 2). Although N-siRNA showed a significant level of fluorescence in the deeper layers of the MCL by 24 h, and almost uniform distribution

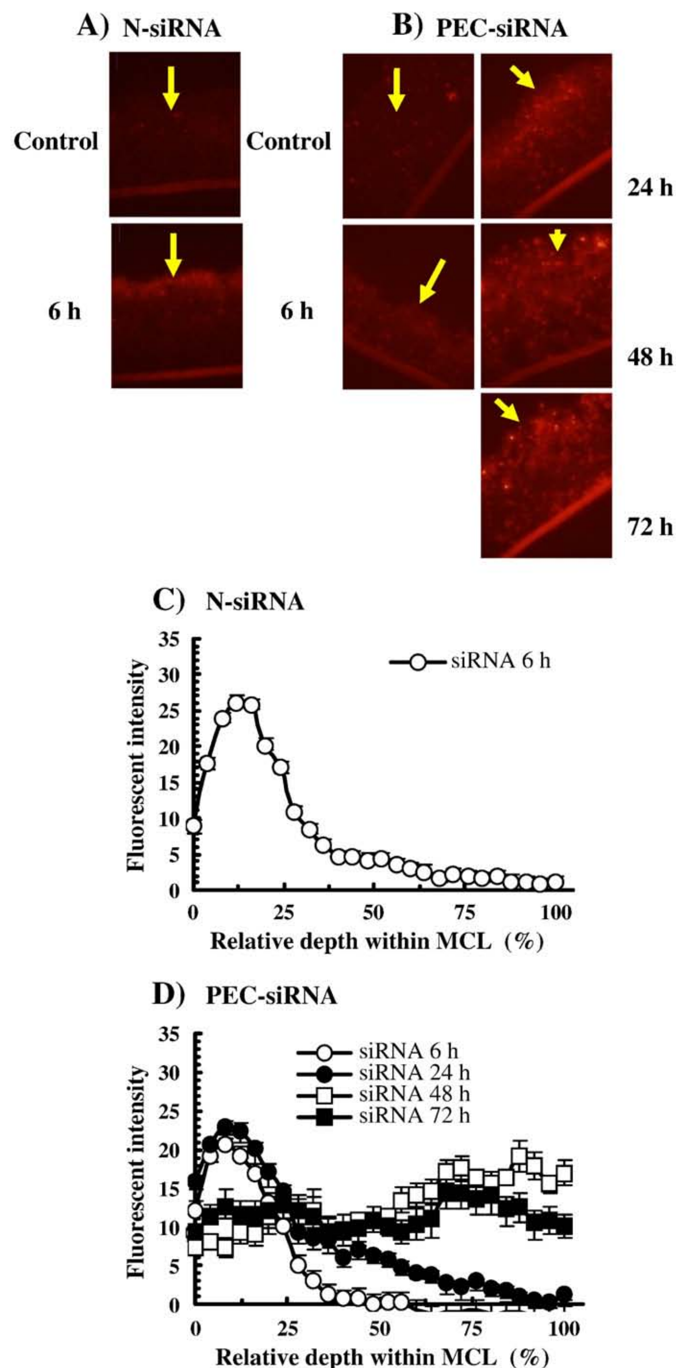

Fig. 2. Penetration/distribution profile of siRNA within the MCLs of DLD-1 cells. MCLs were exposed to 200 pmol of Cy-3 labeled N-siRNA (A) or PEC-siRNA micelles (B) until 72 h and snap-frozen at the end of drug exposure. Twenty micrometer sections were made and siRNA distribution was examined on an inverted fluorescent microscope. Spatial distribution profiles within the MCL after 6 (○), 24 (●), 48(□), and 72(■) h of exposure are shown for N-siRNA and PEC-siRNA in C and D, respectively. The relative net fluorescent intensity was plotted against the distance from the surface of MCL (drug exposure surface indicated by arrow). Data are expressed as mean ± SEM (n = 4).

throughout the layers by 48 h (data not shown), this may have represented the distribution of degraded product instead of the intact Cy3-siRNA molecule due to its instability in the conditioned media as shown by the complete loss of siRNA at 24 h and later (Fig. 1). For PEC-siRNA, distribution was limited to the upper layers of the MCL until 24 h, and the full depth penetration occurred after 48 h, indicating slower penetration compared to N-siRNA (Fig. 2-B and D). The distribution patterns of siRNA within the MCL were also different between naked formulation and PEC-siRNA, i.e., a punctuate pattern with focal areas was apparent for the PEC formulation in contrast to the smooth and uniform distribution of fluorescence for the N-siRNA. Based on the increased stability of siRNA-PEC, the fluorescence signals detected within the MCL were considered to maintain RNA interference effect.

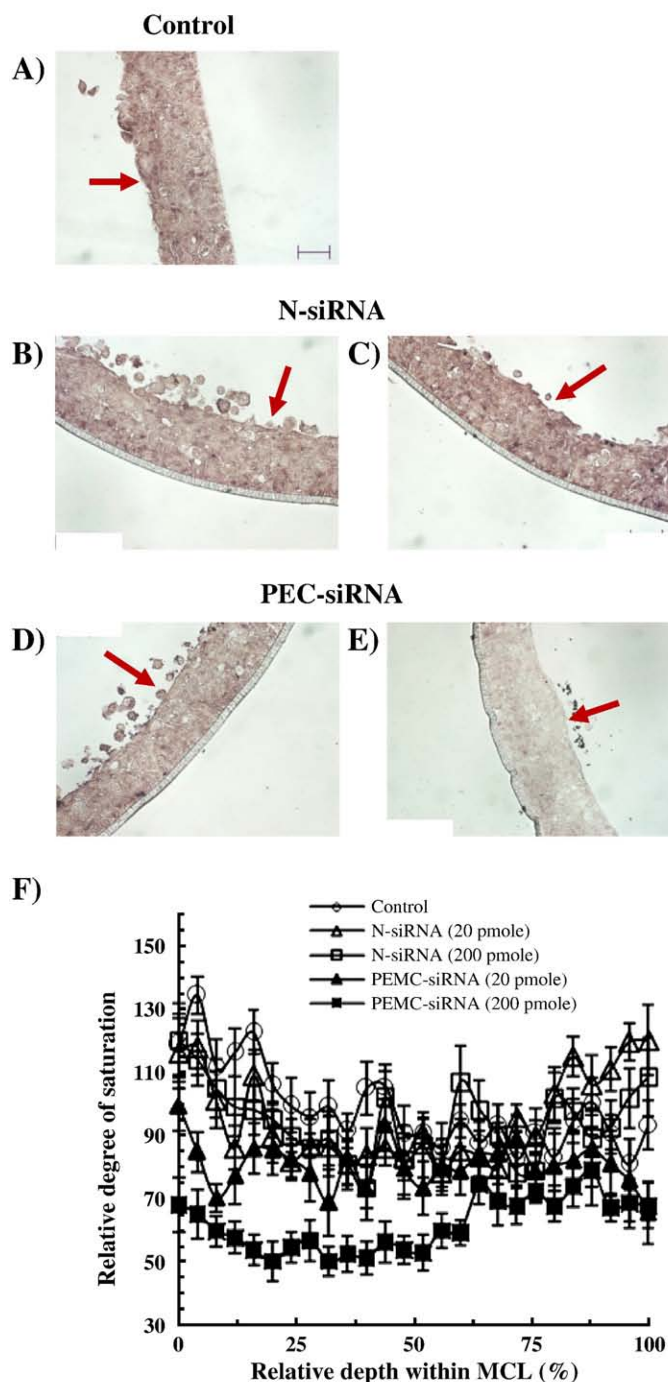

Fig. 3. Changes in VEGF mRNA expression within MCLs of DLD-1cells after exposure to VEGF-siRNA. MCLs were exposed to PBS control (A), VEGF N-siRNA (B and C), or PEC-siRNA (D and E) at 20 pmol (B and D) or 200 pmol (C and E) for 72 h. *In-situ* hybridization was performed on paraffin sections, and the color density was determined using Optimas ver. 6.5 software as described in the Methods. The relative degree of brown color saturation was plotted as a function of depth for control (○), 20 (△, ▲) and 200 pmol (□, ■) of N-siRNA or PEC-siRNA (F). Data are expressed as mean ± SEM ($n=4$). Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. VEGF gene silencing within MCLs of DLD-1 cells

The expression of VEGF mRNA was determined within the layers of DLD-1 MCLs after exposure to N-siRNA and PEC-siRNA targeting VEGF (20 and 200 pmol). No significant changes were observed in the VEGF mRNA levels in MCLs treated with N-siRNA compared to control samples (Fig. 3-A, B and C), indicating the absence of gene silencing effect due to the loss of N-siRNA activity. On the other hand, the VEGF mRNA level decreased significantly compared to the untreated control group (Fig. 3-A, D and E), when MCLs were exposed to PEC-formulated siRNA. The degree of VEGF gene silencing within the MCL was found to be dose dependent showing greater suppression at 200 pmol than at 20 pmol.

3.4. Evaluation of VEGF level after treatment with anti-VEGF siRNA

The VEGF levels within the layers of the MCL (Fig. 4) and in the media (Fig. 5) were evaluated after exposure to N-siRNA and PEC-siRNA at 20 and 200 pmol for 72 h. The distribution of VEGF protein was higher toward the top or bottom layers of the MCL in the untreated samples (Fig. 4-A). N-siRNA failed at either dose level to exert any significant effect on the VEGF protein level within the MCL (Fig. 4-B and C), while PEC-siRNA significantly decreased the VEGF level (Fig. 4-D and E). The effect was dose-dependent, i.e., at higher dose levels (200 pmol), VEGF expression was almost abolished in the MCL. The level of VEGF secreted into the media of the bottom chamber also showed a significant decrease in response to PEC-siRNA treatment, but not to N-siRNA treatment (Fig. 5).

4. Discussion

Small interfering molecules of RNA carrying specific and potent gene silencing ability have been developed rapidly into potential therapeutics for human diseases including cancers [14,15]. Among the many potential targets in cancer, VEGF has been studied as a gene silencing target for tumor growth inhibition secondary to its antiangiogenic effect [13,23,34]. Unlike antibodies and other small molecules inhibiting angiogenesis via receptor binding on the plasma membrane or dysregulation of down-stream signalings in endothelial cells, siRNA for VEGF should be delivered into tumor parenchymal tissue and taken up by VEGF-secreting tumor cells. Therefore, poor serum stability and large molecular weight necessitate a special drug delivery system to ensure its antitumor potential. A wide variety of non-viral delivery systems have been suggested for siRNA such as neutral liposomes [11], cationic cardiolipin liposomes [12], cationized gelatin [13], and atelocollagens [14]. Although a stabilization effect has been obtained, these studies have utilized local administration of siRNA formulation where intratumoral delivery from systemic circulation is not an issue. Polyelectrolyte complex micelles formulated using PEG-conjugation and PEI incorporation (VEGF siRNA-PEG/PEI PEC micelles) have been successfully applied for systemic delivery of VEGF siRNA [23]. The significant inhibition of tumor growth has been attributed to decreases in microvessel density and intratumoral VEGF levels after systemic administration, as previously shown in the study. The efficacy was obtained, however, by multiple systemic dosing in an early tumor model, strongly indicating the presence of drug distribution barrier in tumors *in-vivo*. Hence, in the present study, the intratumoral delivery profile of the siRNA complex was studied in detail using an *in-vitro* solid tumor model, in which tissue

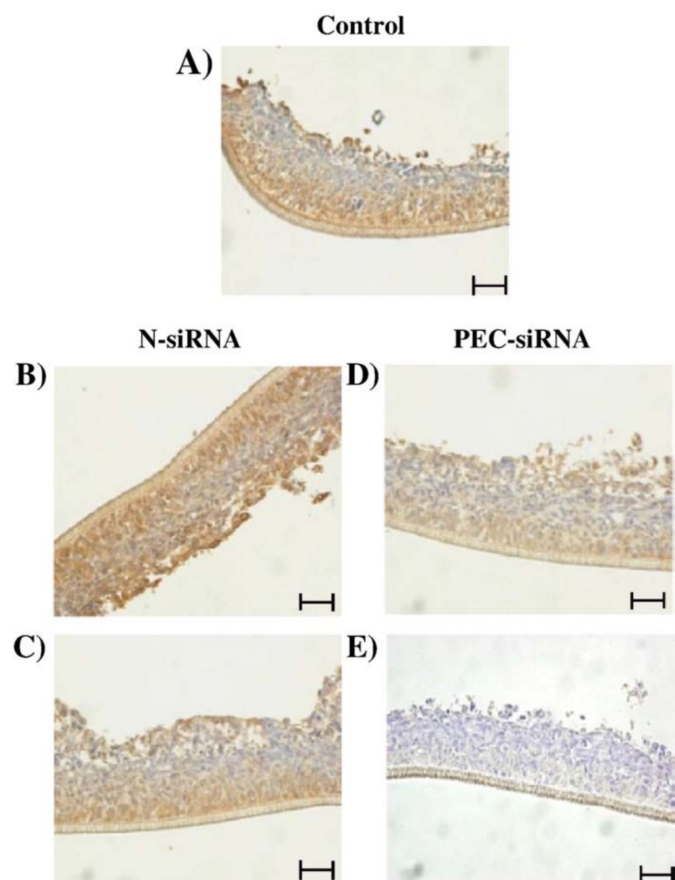

Fig. 4. Changes in the VEGF protein level within the MCLs of DLD-1cells after exposure to anti-VEGF siRNA. MCLs were exposed to PBS control (A), VEGF N-siRNA (B and C), or PEC-siRNA (D and E) at 20 pmol (B and D) or 200 pmol (C and E) for 72 h. Immunohistochemistry was done on paraffin sections. Scale bar = 100 µm.

penetration/distribution and ensued gene silencing could be monitored spatially.

VEGF siRNA was successfully stabilized by PEG conjugation, as shown by the significant amount remaining in 50% serum challenge conditions at 16 h [22]. Although PEI alone does not provide enough stabilizing effect in the siRNA, when complexed with pegylated siRNA, PEI further improves the stability of siRNA, showing no loss until 48 h [22]. In the current expreriment, we confirmed the stability of PEC micelle formulations in MCL conditioned media for a drug exposure

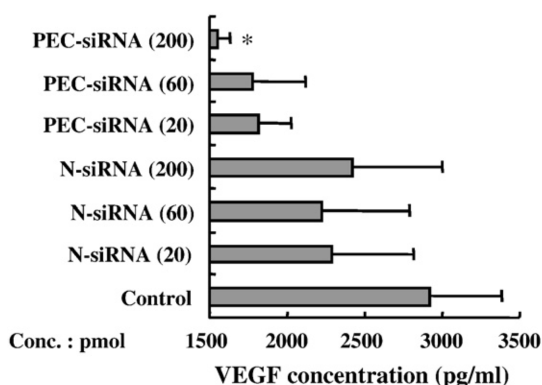

Fig. 5. The level of VEGF protein secreted in the bottom chamber of MCL before and after exposure to VEGF siRNA. MCLs were exposed to PBS control, N-siRNA or PEC VEGF-siRNA at 20, 60, and 200 pmol for 72 h. The level of VEGF in the conditioned media was analyzed by ELISA as described in methods. Data are expressed as mean ± SEM ($n = 4$). *Significantly different from control, $P < 0.05$.

duration of 72 h (Fig. 1). PEI interacts with siRNA molecules electrochemically, increases transfection efficiency in-vivo and *in-vitro*, and induces an endosomal escape effect [10,35]. On the other hand, toxicity is the main limitation of PEI as a gene carrier, therefore, other cationic core forming agents such as KALA fusigenic peptide and hyaluronic acid have also been studied [36,37].

PEC-siRNA micelles showed limited accumulation within the upper 25% of the MCL layers at 24 h, whereas a similar accumulation profile was shown for N-siRNA after only 6 h exposure, indicating that N-siRNA penetrated top layers relatively faster than PEC siRNA micelles. However, a significant decrease in the m-RNA level was observed only for PEC-siRNA micelles, not for N-siRNA (Fig. 3), indicating the poor transfection efficiency of N-siRNA [23]. PEC-siRNA micelles showed a unique distribution pattern with several focal areas over the cell layers (Fig. 2B), which may represent the penetration path and cellular accumulation of PEC micelles. On the other hand, N-siRNA showed more or less uniformly scattered fluorescence distribution (data not shown), which may be attributable to its degradation into small particles and its random distribution without focal cellular accumulation. These results suggest that PEC-siRNA may retain its size and transfection efficiency while penetrating several cell layers in avascular tumor regions after extravasation from the systemic circulation.

We showed in multicellular layer system the rate and extent of avascular distribution of PEC siRNA in relation to VEGF silencing effect spatially (Figs. 2 and 3). The distribution ratio was calculated as the ratio of average fluorescence in the top 20% vs that in the bottom 20% of the MCL (approx 15 –17 cell layers). The initial accumulation of PEC siRNA micelles in the top layers had a distribution ratio of 6.9 to 8.6 until 24 h, which decreased to 0.9–1.1 after 48 h (Table 1). These data suggest that at least 48 h may be required for deep penetration of non-viral delivery systems of this kind. Hence, 48 h-stability may be the minimum requirement in the design of an efficient delivery system of siRNA targeting cancer cells distant from the vasculartjure. This consideration is even more important given the fact that VEGF is more actively secreted by hypoxic cells that exist in remote regions far away from vasculature acting as an endothelial growth factor, at the same time, as an autocrine survival factor [18–21,38].

When PEC siRNA micelles were given systemically in the previous study, a 5 fold larger dose was required to show similar efficacy with local administration (1500 pmol × 5 doses vs 500 pmol × 3 doses), suggesting the need for improvement in systemic siRNA delivery [23]. EPR effect may increase delivery of DDS from the systemic circulation to the periphery (adjacent to vessels) of the tumor tissue, but diffusive or convectional movement of molecules into avascular regions of such tumors is very limited due to high interstitial fluid pressure in the microenvironment of human solid tumors [27]. Therefore, avascular drug delivery after extravasation may represent another significant obstacle to chemotherapeutic efficacy for large molecules, including antibodies, oligonucleotides, and siRNAs. Considering the fact that hypoxic cells are more resistant to chemotherapy and anti-VEGF therapy can effectively inhibit the survival of these hypoxic cells, further studies are warranted in the development of safe and effective delivery systems for VEGF siRNA. Studies of the distribution (pharmacokinetic)-effect (phamacodynamic) relationship using *in-vitro* solid tumor model, including MCL models, may provide clinically relevant data with respect to selection of the most promising formulations.

Table 1
Distribution ratio[a] of siRNA in MCLs of DLD-1 cells.

Exposure	N-siRNA	PEC-siRNA
6 h	12.5 ± 3.8	6.9 ± 3.6
24 h	NA	8.6 ± 4.3
48 h	NA	1.1 ± 0.8
72 h	NA	0.9 ± 0.4

[a] Distribution ratio is the ratio of the average fluorescence in the top 20% vs the bottom 20% of the layers of the MCL thickness (Fig. 2-C and D).

5. Conclusion

We demonstrated that full penetration of PEC anti-VEGF siRNA took place after 48 h in the MCL model of human solid tumor. The PEC formulation of VEGF siRNA effectively suppressed gene expression throughtout the cell layers of the MCL. Our data support the previous finding that the PEC formulation of VEGF siRNA significantly inhibits a tumor growth in xenograft model. Further studies are warranted in the development of safe (non toxic) DDS, which can ensure the stability of the loaded siRNA, as well as, the effective delivery to the major target area distant from the vasculature so that antitumor efficacy can be obtained with fewer and lower doses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.03.009.

References

[1] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (6836) (2001) 494–498.
[2] R.K. Leung, P.A. Whittaker, RNA interference: from gene silencing to gene-specific therapeutics, Pharmacol. Ther. 107 (2) (2005) 222–239.
[3] J.S. Kang, Y.N. Yum, E. Han, J.H. Kim, E.M. Lee, D.-Y. Ryu, Y.H. Kim, S.H. Yang, S.H. Kim, S.N. Park, Evaluation of potential biomarkers for thioacetamide-induced hepatotoxicity using siRNA, Biomol. Ther. 16 (3) (2008) 197–202.
[4] E. Song, S.K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, J. Lieberman, RNA interference targeting Fas protects mice from fulminant hepatitis, Nat. Med. 9 (3) (2003) 347–351.
[5] T.C. Karagiannis, A. El-Osta, RNA interference and potential therapeutic applications of short interfering RNAs, Cancer Gene Ther. 12 (10) (2005) 787–795.
[6] J.C. Cheng, T.B. Moore, K.M. Sakamoto, RNA interference and human disease, Mol. Genet. Metab. 80 (1–2) (2003) 121–128.
[7] R.C. Ryther, A.S. Flynt, J.A. Phillips, 3rd, J.G. Patton, siRNA therapeutics: big potential from small RNAs, Gene Ther. 12 (1) (2005) 5–11.
[8] J.M. Layzer, A.P. McCaffrey, A.K. Tanner, Z. Huang, M.A. Kay, B.A. Sullenger, In vivo activity of nuclease-resistant siRNAs, RNA 10 (5) (2004) 766–771.
[9] S. Choung, Y.J. Kim, S. Kim, H.O. Park, Y.C. Choi, Chemical modification of siRNAs to improve serum stability without loss of efficacy, Biochem. Biophys. Res. Commun. 342 (3) (2006) 919–927.
[10] T.G. Park, J.H. Jeong, S.W. Kim, Current status of polymeric gene delivery systems, Adv. Drug Deliv. Rev. 58 (4) (2006) 467–486.
[11] C.N. Landen Jr., A. Chavez-Reyes, C. Bucana, R. Schmandt, M.T. Deavers, G. Lopez-Berestein, A.K. Sood, Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery, Cancer Res. 65 (15) (2005) 6910–6918.
[12] A. Pal, A. Ahmad, S. Khan, I. Sakabe, C. Zhang, U.N. Kasid, I. Ahmad, Systemic delivery of Raf siRNA using cationic cardiolipin liposomes silences Raf-1 expression and inhibits tumor growth in xenograft model of human prostate cancer, Int. J. Oncol. 26 (4) (2005) 1087–1091.
[13] G. Matsumoto, T. Kushibiki, Y. Kinoshita, U. Lee, Y. Omi, E. Kubota, Y. Tabata, Cationized gelatin delivery of a plasmid DNA expressing small interference RNA for VEGF inhibits murine squamous cell carcinoma, Cancer Sci. 97 (4) (2006) 313–321.
[14] Y. Takei, K. Kadomatsu, Y. Yuzawa, S. Matsuo, T. Muramatsu, A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics, Cancer Res. 64 (10) (2004) 3365–3370.
[15] A. de Fougerolles, H.P. Vornlocher, J. Maraganore, J. Lieberman, Interfering with disease: a progress report on siRNA-based therapeutics, Nat. Rev. Drug Discov. 6 (6) (2007) 443–453.
[16] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. U S A 92 (16) (1995) 7297–7301.
[17] M. Izquierdo, Short interfering RNAs as a tool for cancer gene therapy, Cancer Gene Ther. 12 (3) (2005) 217–227.
[18] R.S. Kerbel, Improving conventional or low dose metronomic chemotherapy with targeted antiangiogenic drugs, Cancer Res. Treat 39 (4) (2007) 150–159.
[19] F. Fan, J.S. Wey, M.F. McCarty, A. Belcheva, W. Liu, T.W. Bauer, R.J. Somcio, Y. Wu, A. Hooper, D.J. Hicklin, L.M. Ellis, Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells, Oncogene 24 (16) (2005) 2647–2653.
[20] D.J. Hicklin, L.M. Ellis, Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis, J. Clin. Oncol. 23 (5) (2005) 1011–1027.
[21] M. Calvani, D. Trisciuoglio, C. Bergamaschi, R.H. Shoemaker, G. Melillo, Differential involvement of vascular endothelial growth factor in the survival of hypoxic colon cancer cells, Cancer Res. 68 (1) (2008) 285–291.
[22] S.H. Kim, J.H. Jeong, S.H. Lee, S.W. Kim, T.G. Park, PEG conjugated VEGF siRNA for anti-angiogenic gene therapy, J. Control Release 116 (2) (2006) 123–129.
[23] S.H. Kim, J.H. Jeong, S.H. Lee, S.W. Kim, T.G. Park, Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer, J. Control Release 129 (2) (2008) 107–116.
[24] K. Greish, Enhanced permeability and retention of macromolecular drugs in solid tumors: a royal gate for targeted anticancer nanomedicines, J. Drug Target 15 (7–8) (2007) 457–464.
[25] A.K. Iyer, G. Khaled, J. Fang, H. Maeda, Exploiting the enhanced permeability and retention effect for tumor targeting, Drug Discov. Today 11 (17–18) (2006) 812–818.
[26] O. Tredan, C.M. Galmarini, K. Patel, I.F. Tannock, Drug resistance and the solid tumor microenvironment, J. Natl. Cancer Inst. 99 (19) (2007) 1441–1454.
[27] A.I. Minchinton, I.F. Tannock, Drug penetration in solid tumours, Nat Rev. Cancer 6 (8) (2006) 583–592.
[28] G. Fracasso, M. Colombatti, Effect of therapeutic macromolecules in spheroids, Crit. Rev. Oncol. Hematol. 36 (2–3) (2000) 159–178.
[29] B. Desoize, Contribution of three-dimensional culture to cancer research, Crit. Rev. Oncol. Hematol. 36 (2–3) (2000) 59–60.
[30] K.O. Hicks, F.B. Pruijn, J.R. Sturman, W.A. Denny, W.R. Wilson, Multicellular resistance to tirapazamine is due to restricted extravascular transport: a pharmacokinetic/pharmacodynamic study in HT29 multicellular layer cultures, Cancer Res. 63 (18) (2003) 5970–5977.
[31] A.M. Al-Abd, J.H. Lee, S.Y. Kim, N. Kun, H.J. Kuh, Novel application of multicellular layers culture for in situ evaluation of cytotoxicity and penetration of paclitaxel, Cancer Sci. 99 (2) (2008) 423–431.
[32] L.F. Brown, K.T. Yeo, B. Berse, T.K. Yeo, D.R. Senger, H.F. Dvorak, L. van de Water, Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing, J. Exp. Med. 176 (5) (1992) 1375–1379.
[33] M.Y. Lee, W.K. Ju, J.H. Cha, B.C. Son, M.H. Chun, J.K. Kang, C.K. Park, Expression of vascular endothelial growth factor mRNA following transient forebrain ischemia in rats, Neurosci. Lett. 265 (2) (1999) 107–110.
[34] H. Guan, Z. Zhou, H. Wang, S.F. Jia, W. Liu, E.S. Kleinerman, A small interfering RNA targeting vascular endothelial growth factor inhibits Ewing's sarcoma growth in a xenograft mouse model, Clin. Cancer Res. 11 (7) (2005) 2662–2669.
[35] A. Gautam, C.L. Densmore, B. Xu, J.C. Waldrep, Enhanced gene expression in mouse lung after PEI-DNA aerosol delivery, Mol. Ther. 2 (1) (2000) 63–70.
[36] S.H. Lee, S.H. Kim, T.G. Park, Intracellular siRNA delivery system using polyelectrolyte complex micelles prepared from VEGF siRNA-PEG conjugate and cationic fusogenic peptide, Biochem. Biophys. Res. Commun. 357 (2) (2007) 511–516.
[37] H. Lee, H. Mok, S. Lee, Y.K. Oh, T.G. Park, Target-specific intracellular delivery of siRNA using degradable hyaluronic acid nanogels, J. Control Release 119 (2) (2007) 245–252.
[38] M.P. Barr, D.J. Bouchier-Hayes, J.J. Harmey, Vascular endothelial growth factor is an autocrine survival factor for breast tumour cells under hypoxia, Int. J. Oncol. 32 (1) (2008) 41–48.