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Enhanced Growth Inhibition of Hepatic Multicellular Tumor Spheroids by Lactosylated Poly(ethylene glycol)-siRNA Conjugate Formulated in PEGylated Polyplexes

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PEGylated polyplexes (lac-PEGylated polyplexes) composed of poly(L-lysine) and lactosylated poly(ethylene glycol)-small interfering RNA conjugate, which inhibits the RecQL1 gene product, were revealed to show an appreciable growth inhibition of multicellular HuH-7 spheroids (human hepatocarcinoma cell lines) for up to 21 days (IC_{50} = 6 nM); this system used as an in vitro three-dimensional (3D) model mimicking the in vivo biology of tumors. The PEGylated polyplexes thus prepared had a size of approximately 110 nm with clustered lactose moieties on their periphery as targeting ligands for the asialoglycoprotein-receptor-expressing HuH-7 cells. In contrast, OligofectAMINE/siRNA (cationic lipoplex) was observed to have almost no growth-inhibitory effect against HuH-7 spheroids, even though the lipoplex showed a stronger growth-inhibitory effect than the lac-PEGylated polyplexes on conventional monolayer-cultured HuH-7 cells. The FITC-tagged conjugate in the lac-PEGylated polyplexes showed smooth penetration into the HuH-7 spheroids compared with that in the lipoplexes, as observed by confocal fluorescence-scanning microscopy. This indicates that the small size of approximately 100 nm and the reduced nonspecific interaction due to the nonionic and hydrophilic lactosylated PEG layer contributes to the smooth penetration of the PEGylated polyplexes into the spheroid interior, eventually facilitating their uptake into the cells composing the spheroids. Cellular apoptosis indicating programmed cell death was also observed in the HuH-7 spheroids treated with the PEGylated polyplexes, revealing that the observed growth inhibition was indeed induced by the RNAi of the RecQL1 siRNA. These data suggest that the smart PEGylated polyplexes can indeed penetrate into the multiple cell layers of 3D tumor masses in vivo, exerting therapeutic effects through the RNAi.

Introduction

The targeted delivery of small interfering RNAs (siRNAs)[1] is one of the major challenges in the field of cancer therapy through RNA interference (RNAi),[2] because siRNAs often tend to show low stability against enzymatic degradation, low permeability across the cell membrane, and preferential liver and renal clearance.[3] Therefore, the therapeutic value of siRNAs under in vivo conditions is largely dependent on the development of effective carrier systems which achieve modulated disposition in the body intravenously and accumulation in tumor tissues by enhanced permeability and retention (EPR) effect.[4] A promising strategy in this regard is the combination of PEGylation and carrier, namely a "smart" siRNA carrier (PEGylated polyplex) formulated through the supramolecular assembly (electrostatic interactions) of poly(L-lysine) (PLL) and lactosylated poly(ethylene glycol)-siRNA conjugate (Lac-PEG-siRNA) (Figure 1).[5] These smart PEGylated polyplexes, which have a size of approximately 100 nm, showed the high biocompatibility and enzymatic tolerability due to their segregated polyion complex core surrounded by a palisade of flexible and hydrophilic PEG layers. In particular, these smart PEGylated polyplexes with clustered lactose moieties on their periphery were successfully transported into the monolayer-cultured hepatic tumor cells by mediation of the asialoglycoprotein (ASGP) re-

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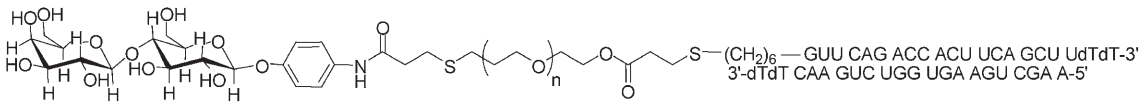

Figure 1. Chemical structure of the Lac-PEG-siRNA conjugate.

ceptor, inducing significant gene silencing of firefly luciferase (reporter gene) expression in monolayer-cultured HuH-7 cells at an extremely low siRNA concentration ($IC_{50} = 1.3$ nM). Therefore, the combination of this smart PEGylated polyplex system and a proper therapeutic siRNA is a promising approach to the creation of a systemic siRNA delivery system for the cancer therapy.

In this regard, siRNA-targeting DNA helicases are of particular interest. DNA helicases have recently been recognized to play important roles in DNA replication, recombination, repair, and transcription. Among many kinds of DNA helicases in living cells, the RecQ helicase family has been shown to have unique properties which are apparently involved in maintaining genomic stability. In humans, the RecQ helicase family has five members: RecQL1, BLM, WRN, RTS, and RecQL5.[6] BLM, WRN, and RTS are causative genes of Bloom syndrome, Werner syndrome, and a subset of Rothmund-Thomson syndrome, respectively, all of which are known to be recessive genetic disorders. Although RecQL1 is not yet known to have a relationship with any human disease, recent findings suggest that it is involved in the maintenance of the human genome.[7] LeRoy et al. have reported that RecQL1 helicase has Holliday junction branch migration activity, and the down-regulation of RecQL1 mRNA by RNAi resulted in an increase in sister chromatid exchange in human cells,[8] suggesting that RecQL1 helicase maintains the human genome by suppressing chromosomal recombination in the S phase. In addition, the RecQL1 protein was up-regulated by mitogenic stimulation or viral transformation in human B-lymphocytes,[9] suggesting that RecQL1 helicase is involved in genomic stabilization in growing cells such as cancer cells. Accordingly, RecQL1 helicase might be a good molecular target in cancer therapy.[10]

We would like to report herein, the significant and prolonged growth inhibition of hepatic multicellular tumor spheroids (MCTSs) by smart PEGylated polyplexes composed of PLL and Lac-PEG-siRNA conjugate bearing a RecQL1-siRNA segment. Note that MCTSs provide an in vivo tumor microenvironment characterized by high cell density, elevated interstitial pressure, hypoxia, and the existence of cell-cell contacts and a tumor extracellular matrix (ECM).[11] Apparently, these environmental factors play key roles in the diffusion, penetration, and growth-inhibitory effects of the siRNA-carriers in solid tumors in vivo. Therefore, using MCTSs, the efficacy of the siRNA delivery systems, including PEGylated polyplexes and lipoplexes, can be simply evaluated from the direct observation of the MCTS size; this method reflects the environmental factors characteristic of tumors and the direct gene silencing ability of the siRNA-carriers.

Results and Discussion

Design of the smart PEGylated polyplexes

Our strategy of formulating PEGylated polyplexes is based on the novel conjugation of siRNA with lactosylated PEG (Lac-PEG-siRNA), followed by complexation with poly(L-lysine) (PLL). The PEG-siRNA conjugates were synthesized according to our previously reported method;[5] the Michael addition of α-lactosyl-ω-acryl-PEG toward the 5′-thiol modified sense RNA to obtain Lac-PEG-single stranded RNA conjugate, followed by annealing with antisense RNA to prepare the Lac-PEG-siRNA conjugate through hybridization. A nonlactosylated conjugate, Ace-PEG-siRNA, was also prepared from the α-acetal-ω-acryl-PEG. The lactosyl-(lac-PEGylated polyplex) and nonlactosyl-(ace-PEGylated polyplex) PEGylated polyplexes were then prepared at an N/P ratio of 1 (=[amino group in polycation]/[phosphate group in siRNA segment]) by mixing the siRNA-PEG conjugates and PLL (degree of polymerization (DP) = 40, 100, or 460). The diameter of the PEGylated polyplex was determined to be approximately 110 nm by TEM.[5]

Growth inhibition of monolayer-cultured tumor cells by the PEGylated polyplexes

The elevation of RecQL1 expression has been positively correlated with various cancer cells, and the depletion of RecQL1 by siRNA complementary to RecQL1 mRNA dramatically inhibited cell proliferation and induced apoptosis in vitro and in vivo.[10] In contrast, the inhibition of the RecQL1 gene product in normal cells by RecQL1 siRNA induces no effect on cell proliferation, suggesting that RecQL1 siRNA is a potential therapeutic tool specific to the molecular targeting of cancer cells. To characterize the growth-inhibitory effect of the PEGylated polyplex system containing RecQL1 siRNA, an MTT assay was done using monolayer-cultured HuH-7 cells (human hepatoma cell) possessing asialoglycoprotein (ASGP) receptors, which recognize and internalize compounds bearing terminal lactose moieties.[12] As seen in Figure 2, almost no growth inhibition was observed for siRNA alone and conjugate alone even at a siRNA concentration of 150 nM in the presence of 10% fetal bovine serum (FBS). On the contrary, lac-PEGylated polyplex with PLL (DP = 100) showed 20% growth inhibition ($P^* < 0.05$) at 150 nM conjugate concentration. These results suggest that the lack of any growth-inhibitory effect on HuH-7 cells for free siRNA and free Lac-PEG-siRNA conjugate may be ascribed to the enzymatic degradation of the siRNA in the medium and to the impaired diffusivity of the negatively charged and hydrophilic free siRNA and Lac-PEG-siRNA conjugate through the negatively charged cell membrane. Significant growth inhibition was observed for the siRNA formulated with commercially

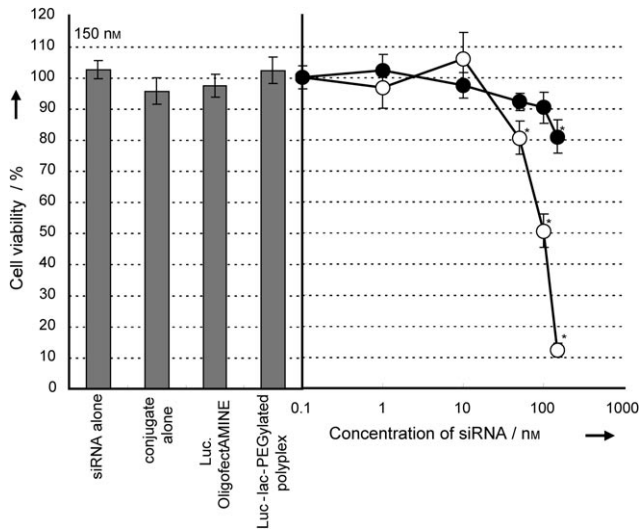

Figure 2. Growth inhibitory effect of the lac-PEGylated polyplexes (closed circles) and lipoplexes (open circles) from RecQL1 siRNA on monolayer-cultured HuH-7 cells. The cell viability was determined by means of an MTT assay after 96 h of incubation. The indicated concentrations of siRNA and conjugate are the final ones in the total transfection volume (250 μL). The plotted data are averages of triplicate experiments ± SD. The data points marked with asterisks are statistically significant compared with the mock data (buffer-treated cells) ($P^* < 0.05$).

available cationic lipid reagents, such as OligofectAMINE (lipoplex) (87% inhibition, $P^* < 0.05$). The lipoplex, which is cationic in character, may strongly interact with the negatively charged cell membrane leading to an appreciable increase in cellular uptake. It should be noticed that the lac-PEGylated polyplex and lipoplex, including luciferase-siRNA as a nontargeted sequence, induced no growth inhibition, strongly suggesting that the observed inhibitory effect against monolayer-cultured HuH-7 cells indeed occurred in a sequence-specific manner through RNAi based on the RecQL1-siRNA.

Growth inhibition of multicellular tumor spheroids (MCTS) by the PEGylated polyplexes

Although the efficacy of the siRNA formulated in the carrier systems in vitro has been generally evaluated by means of monolayer-cultured tumor cells, in vivo results to date have not always been in line with in vitro ones even if the carriers accumulated considerably in the tumor tissues because of the EPR effect. One plausible reason for this discrepancy may be that the monolayer assay only reflects the acute efficacy of the first several days, possibility overlooking the delayed or sustained siRNA action appearing at later stages. Furthermore, the diffusivity of the carriers into the 3D tumor tissue may be crucial in determining the in vivo efficacy, because hypoxic cells that are distant from blood vessels are relatively resistant to chemotherapy, causing the regrowth of the tumor; that is, there are tumor stem cells in the hypoxic regions of some tumors.[13] Therefore, alternatives to in vivo studies (animal experimentations), that is, appropriate in vitro models of in vivo solid tumors, are required to evaluate the prolonged efficacy of siRNA delivery systems, and to properly determine the diffusivity of siRNA-carriers into 3D tumor masses. Worth noting in this regard is the MCTS, which can be maintained in culture medium for many weeks with the physiological characteristics (microenvironment conditions) of in vivo 3D tumor tissues, such as high cell density, elevated interstitial pressure, hypoxia, the existence of cell-cell contacts, and ECM.[11] Indeed, the efficacy of gene delivery by cationic polyplexes and lipoplexes is limited because of their poor penetration ability into MCTSs.[14] Thus, MCTSs were used in this study as 3D in vitro tumor models for screening the growth-inhibitory effect of the siRNAs and their penetration ability into the spheroid interior.

RecQL1 siRNA-mediated growth inhibition of HuH-7 spheroids was assessed under the condition of prolonged culturing (up to 21 days). An HuH-7 spheroid with approximately 100 μm (75–100 μm) in diameter was initially used as the in vitro tumor model, because the maximum distance between the capillary blood vessels within avascular solid tumors is believed to be 200 μm or less.[15] As seen in Figure 3, no growth-inhibitory effect was observed for the siRNA alone or the Lac-PEG-siRNA conjugate alone, even at siRNA concentrations as high as 100 nM. These findings are consistent with the results obtained from the MTT assay using monolayer-cultured HuH-7 cells (Figure 2). In contrast, both the ace-PEGylated and lac-PEGylated polyplexes revealed a significant growth-inhibitory

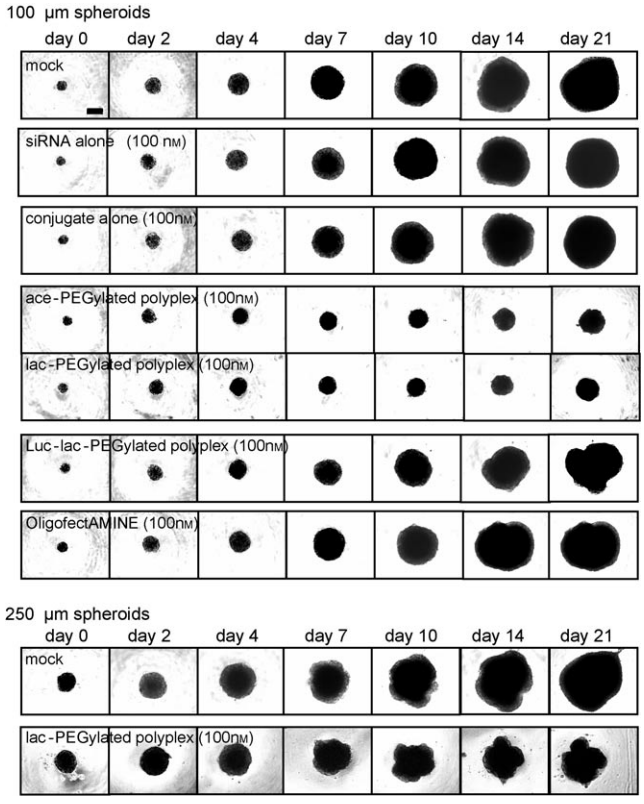

Figure 3. Phase-contrast images of the HuH-7 spheroids with an initial diameter of 100 μm and 250 μm (bar = 100 μm) treated with siRNA alone, Lac-PEG-siRNA conjugate alone, ace-PEGylated polyplex, lac-PEGylated polyplex, Luc-lac-PEGylated polyplex, and OligofectAMINE at concentrations of 100 nM.

effect in a dose-dependent manner (Figure 4a and b). In particular, the lac-PEGylated polyplexes exerted far more effective growth inhibition than the ace-PEGylated polyplexes at a conjugate concentration of as low as 10 nM; the 50% inhibitory concentration (IC_{50}) was determined to be 6 nM and 25 nM for the lac-PEGylated polyplex and ace-PEGylated polyplex, respectively (Figure 5). This almost fourfold increase in the growth-inhibitory effect exerted by lac-PEGylated polyplex is likely to be due to the facilitated uptake into the HuH-7 cells through an ASGP receptor-mediated endocytosis process.[5] This long-term growth-inhibitory effect (up to 21 days) on the spheroids by the single dose of PEGylated polyplexes added at the beginning is worth noting. The control lac-PEGylated polyplex, which included a siRNA against the firefly luciferase sequence (Luc-lac-PEGylated polyplex), induced almost no growth-inhibitory effect even at a conjugate concentration of 100 nM, strongly suggesting that the observed growth-inhibitory effect of the PEGylated polyplexes on the HuH-7 spheroids indeed occurred in a sequence-specific manner (Figure 3 and 4). Although the lipoplexes showed a higher growth-inhibitory effect than the PEGylated polyplex system on monolayer-cultured HuH-7 cells (Figure 2), almost no growth-inhibitory effect on the HuH-7 spheroids was observed even at a siRNA concentration of 100 nM (Figures 3 and 4a). This is presumably due to the cationic nature of the lipoplexes interacting nonspecifically with the negatively charged cell membrane and ECM, which leads to poor penetration into the HuH-7 spheroids. In addition, the spheroid size did not influence the growth-inhibitory effect of the PEGylated polyplexes; the lac-PEGylated polyplexes showed a significant growth-inhibitory effect on the HuH-7 spheroids with an initial diameter of both 200 (data not shown) and 250 μm (Figure 3), whereas the lipoplexes showed almost no growth-inhibitory effect even at high siRNA concentrations (100 nM).

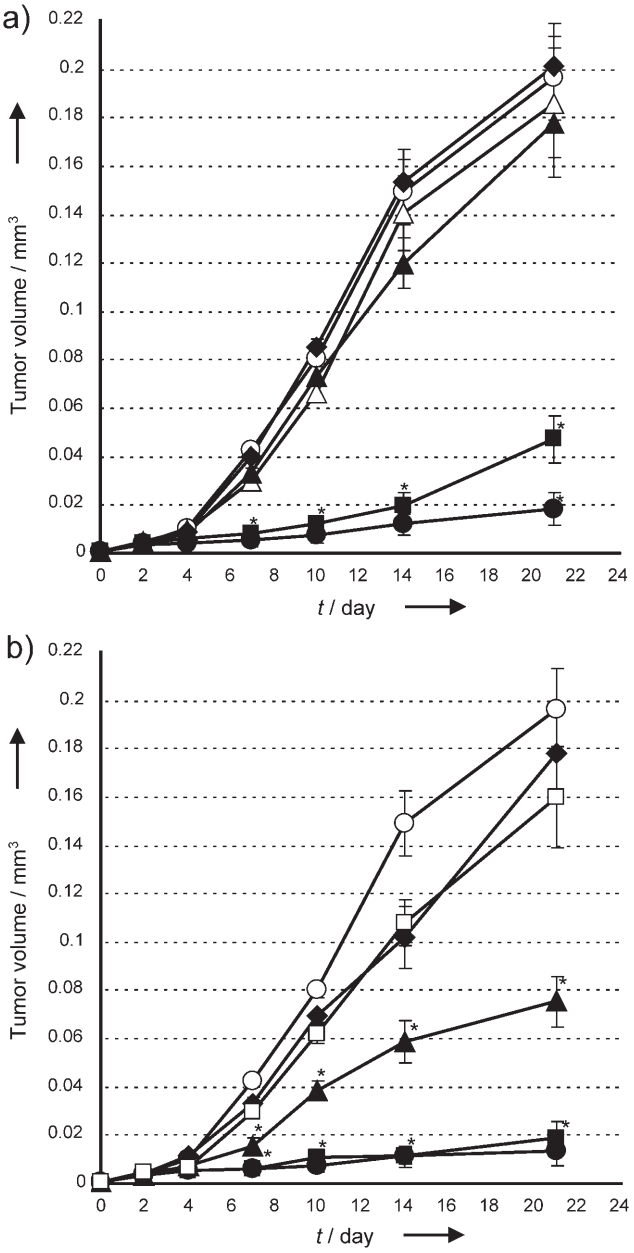

Figure 4. Time-dependent change in the volume of the spheroids treated with a) ace-PEGylated polyplexes and b) lac-PEGylated polyplexes; mock (open circles), OligofectAMINE at [siRNA] = 100 nM (open triangles), Luc-lac-PEGylated polyplex at [conjugate] = 100 nM (open squares), and the PEGylated polyplexes at [conjugate] = 100 nM (closed circles), 50 nM (closed squares), 10 nM (closed triangles), and 1 nM (closed lozenges). The volume of the spheroids was calculated as described in the Experimental Section. The data are averages of five HuH-7 spheroids ± SD. The data points marked with asterisks are statistically significant compared with the mock data (buffer-treated cells) ($P^* < 0.05$).

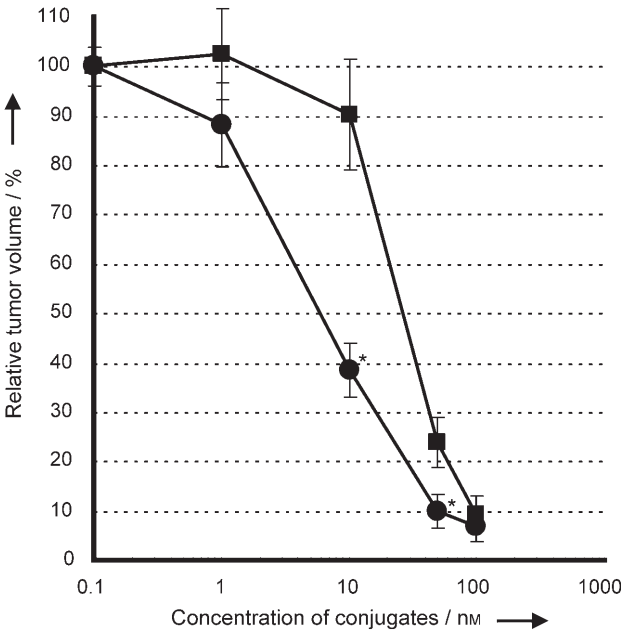

Figure 5. Effect of the complexed PEG-siRNA conjugate concentration on the growth inhibition of the HuH-7 spheroids with an initial diameter of 100 μm (closed squares: ace-PEGylated polyplexes; closed circles: lac-PEGylated polyplexes). The relative spheroid volume as the vertical axis was defined as the ratio of volume of the HuH-7 spheroids treated with PEGylated polyplexes to that of the mock sample on day 21. The data are averages of five HuH-7 spheroids ± SD. The data points for the lac-PEGylated polyplexes marked with asterisks are statistically significant compared with those for the ace-PEGylated polyplexes at the corresponding concentrations ($P^* < 0.05$).

Effect of the PLL length on the growth inhibition of spheroids by the PEGylated polyplexes

The effect of the PLL length on the growth inhibition of spheroids induced by the lac-PEGylated polyplexes was then examined. PLLs with varying DPs (40, 100, or 460) were used to prepare the PEGylated polyplex of the PEG-siRNA conjugate. As can be seen in Figure 6a, a striking effect of the PLL length on the growth inhibition of HuH-7 spheroids was observed at a conjugate concentration of 10 nM; the lac-PEGylated polyplexes prepared from shorter PLL (DP = 40) showed only limited efficacy relative to those from longer PLLs (DP = 100 or 460). Consequently, the IC_{50} was determined to be 7 nM, 6 nM, and 70 nM for the lac-PEGylated polyplexes prepared from PLL with DP = 460, DP = 100, and DP = 40, respectively (Figure 6b). These results indicate that the lac-PEGylated polyplexes formed from shorter PLL (DP = 40) are probably unstable under the extremely dilute conditions because of the critical dissociation phenomenon,[16] resulting in the impaired cellular uptake of the Lac-PEG-siRNA conjugate.

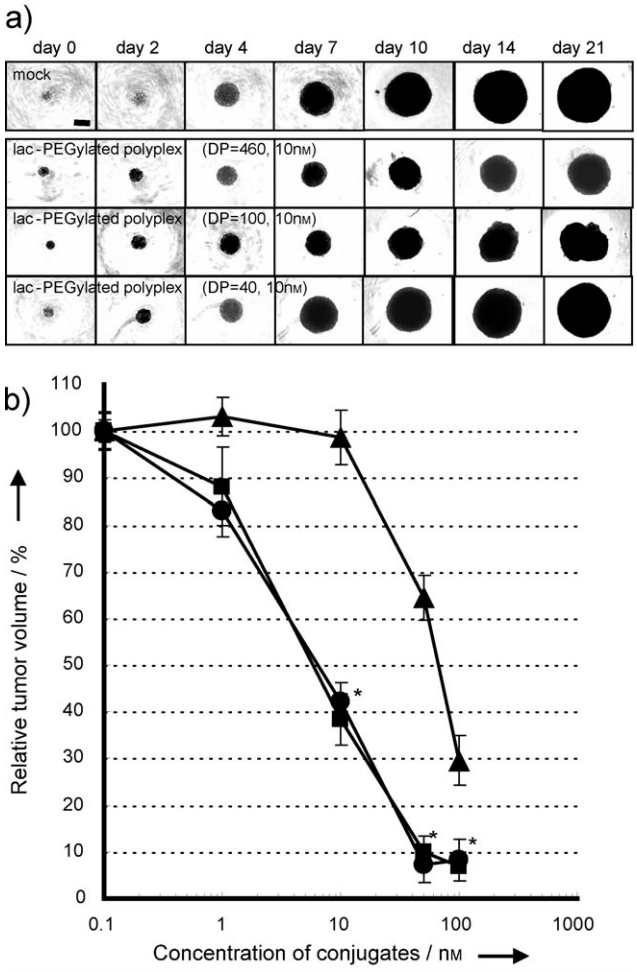

Figure 6. Effect of the PLL length on the growth-inhibitory effect of the lac-PEGylated polyplexes on the HuH-7 spheroids with an initial diameter of 100 μm (bar = 100 μm). a) Phase-contrast images of spheroids treated with the lac-PEGylated polyplexes composed of PLL with varying chain lengths. b) Change in the relative volume of the HuH-7 spheroids on day 21 with various concentrations of lac-PEGylated polyplexes composed of PLL with varying chain lengths (closed triangles: DP = 40; closed squares: DP = 100; closed circles: DP = 460). The relative spheroid volume as the vertical axis was defined as the ratio of volume of the HuH-7 spheroids treated with PEGylated polyplexes to that of the mock sample on day 21. The data are averages of five HuH-7 spheroids ± SD. The data points marked with asterisks are statistically significant compared with those for the lac-PEGylated polyplexes (DP = 40) at the corresponding concentrations ($P^* < 0.05$).

Distribution of the PEGylated polyplexes in multicellular tumor spheroids

To confirm whether or not the lac-PEGylated polyplex can effectively penetrate into the HuH-7 spheroids, 5′-FITC-labeled (fluorescein isothiocyanate) oligodeoxynucleotide (ODN) having the same antisense sequence as the firefly luciferase siRNA was hybridized with the sense firefly luciferase PEG-ODN conjugate to form an FITC-labeled Lac-PEG-dsODN conjugate. The FITC-labeled conjugate was mixed with PLL (DP = 100) at an N/P ratio of 1 to form a PEGylated polyplex with the FITC-label (FITC-PEGylated polyplex). An FITC-labeled lipoplex was also prepared by mixing the OligofectAMINE with FITC-labeled dsODN having the same sequence as the firefly luciferase siRNA. The fluorescence of the FITC-labeled lipoplexes and the FITC-PEGylated polyplexes was observed under a confocal fluorescence-scanning microscope after 48 h of incubation as shown in Figure 7. Most of the fluorescence from the FITC-labeled lipoplexes was seen only at the periphery of the HuH-7 spheroid even after 48 h of incubation (Figure 7a), indicating that the lipoplexes have a poor ability to penetrate into the HuH-7 spheroids, presumably due to the large complex size and the strong interaction with the negatively charged cell membrane and/or ECM.[17] This poor penetration of the lipoplexes into the HuH-7 spheroids obviously has a negative effect on the RecQL1 siRNA-mediated growth inhibition. In sharp contrast, the fluorescence from the FITC-PEGylated polyplexes was observed not only at the periphery but also much

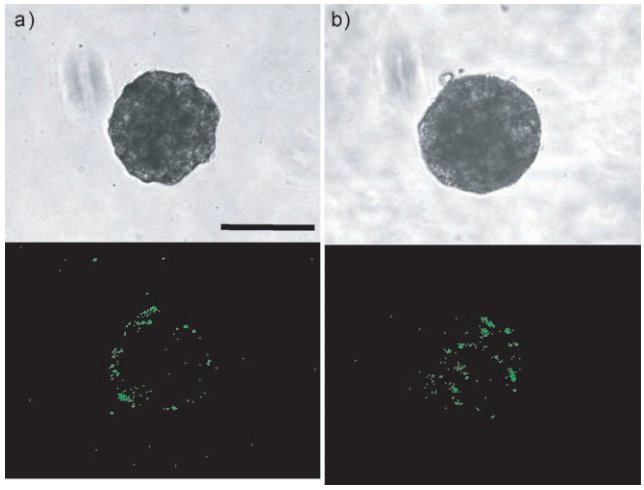

Figure 7. Distribution in the HuH-7 spheroids of the a) FITC-labeled dsODN in the OligofectAMINE lipoplexes (z-axis: 87 μm) and b) FITC-labeled Lac-PEG-dsODN conjugate in the lac-PEGylated polyplexes (z-axis: 79 μm) after 48 h of incubation. Initial diameter of the spheroids: 100 μm (bar = 200 μm).

farther in the interior of the HuH-7 spheroids (Figure 7b) than that of the FITC-labeled lipoplexes after 48 h of incubation (Figure 7b). This result suggests that the small size of approximately 100 nm and the nonionic and hydrophilic PEG shell of the PEGylated polyplexes may reduce the nonspecific interaction of the micelles with the cell membrane and/or ECM, allowing their smooth penetration into the HuH-7 spheroids.[18]

Detection of apoptosis in the HuH-7 spheroids

To confirm whether or not the observed growth-inhibitory effect of the PEGylated polyplexes on the HuH-7 spheroids is due to the induction of cell death, a Live/Dead staining assay was carried out for the HuH-7 spheroids treated with mock, lipoplex (100 nM), and lac-PEGylated polyplex (50 nM), respectively. By means of confocal fluorescence-scanning microscopy, living and dead cells in the HuH-7 spheroids were individually detected as green fluorescence and red fluorescence, respectively. As can be seen in Figure 8, cell death at the center of the mock-treated HuH-7 spheroid was clearly observed on day 7, whereas living cells were only observed at the periphery of the spheroid. The observed cell death in the mock-treated sample was apparently due to necrosis resulting from the insufficient supply of oxygen and nutriment to the spheroid interior, deficiencies which become significant with increasing spheroid size.[11] Note that the spheroids treated with the lipoplexes showed some zones at the periphery containing dead cells on day two, yet after ten days the distribution of dead and living cells was the same as in the mock-treated samples. In contrast, the spheroids treated with lac-PEGylated polyplexes showed continuous cell death at the periphery even after day ten, resulting in the significant death of cells in the spheroids.

In addition to the Live/Dead staining assay, we performed the detection of activated caspase-3 in the HuH-7 spheroids to confirm the induction of apoptosis. The activation of caspase-3 is known to play a central role in the induction of the apoptosis;[19] therefore, caspase-3 is an appropriate marker for measuring apoptosis induced by RecQL1 siRNA. Activated caspase-3 was detected using Magic Red fluorescence probe (MR-$(DEVD)_2$). In the presence of activated caspase-3, the DEVD amino-acid sequence in the MR-$(DEVD)_2$ is cleaved to generate a red fluorescence.[20] As can be seen in Figure 9, apoptotic cells (fluorescence signals) were not observed in the mock-treated spheroids. In contrast, apoptotic cells (fluorescent signals) were observed at the periphery of the spheroids treated with the lipoplex as early as day one. Nevertheless, there was no sign of apoptosis after seven days. It is worth noting that the apoptotic cells were also observed at the periphery of spheroids treated with the lac-PEGylated polyplex, and the number of apoptotic cells increased with prolonged incubation time, from day four to day ten. Note that apoptotic cells were still observed even after ten days. Other spheroids treated with the lac-PEGylated polyplex also showed a similar tendency. These results strongly suggest that the observed growth-inhibitory effect and cell death in the spheroids treated with PEGylated polyplexes are likely to be due to apoptosis induced by RecQL1 siRNA in the long term. In addition, the significant difference in the observation period for apoptotic cells between the PEGylated polyplexes and the lipoplexes may be ascribed to the lower tolerance of the lipoplexes for the culture environment than the PEGylated polyplexes. The appreciable stability under physiological conditions and the uniform size of 100 nm of the PEGylated polyplexes may contribute to their smooth penetration into the spheroids, eventually facilitating the RNAi effect through the continuous uptake into the tumor cells located in the interior and the periphery of the spheroids.

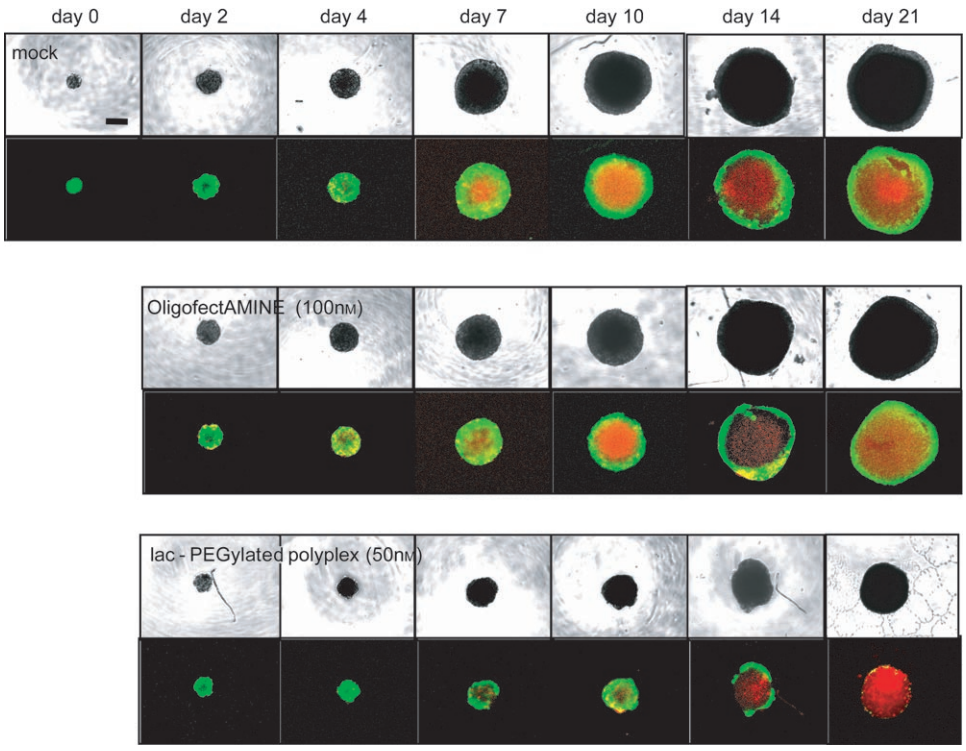

Figure 8. Live/Dead staining assay of the HuH-7 spheroids treated with mock, OligofectAMINE ([siRNA] = 100 nM) and lac-PEGylated polyplexes ([conjugate] = 50 nM) (bar = 100 μm). Living and dead cells emit green and red fluorescences, respectively.

Conclusions

In conclusion, we have demonstrated that MCTSs are useful for evaluating the long-term (up to 21 days) efficacy of siRNA delivery systems (therapeutic value). Note that MCTSs are a versatile in vitro model which can be

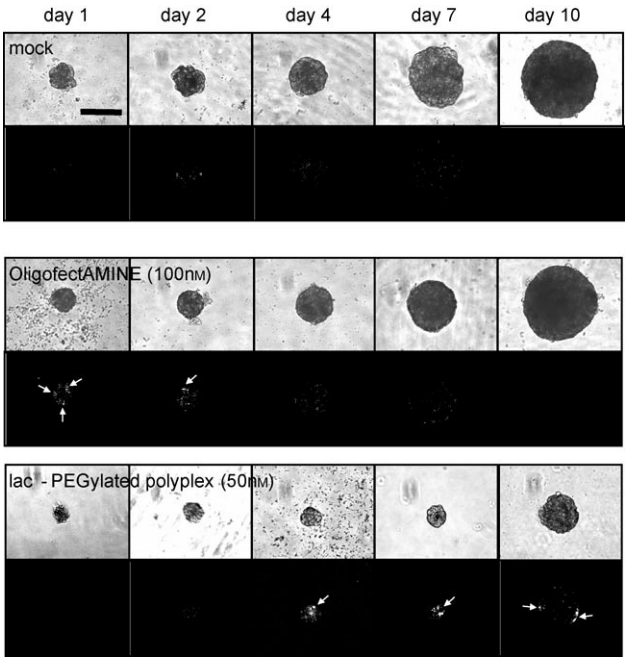

Figure 9. Detection of the activated caspapase-3 as the signal of apoptosis in the HuH-7 spheroids treated with mock, OligofectAMINE ([siRNA] = 100 nM), and lac-PEGylated polyplexes ([conjugate] = 50 nM) (bar = 200 μm).

used to estimate the penetration of carriers into 3D tumor tissues and the effect of the ECM and cell-cell contacts (microenvironment) that could otherwise only be examined in vivo using animal models. Indeed, the lac-PEGylated polyplexes composed of the Lac-PEG-siRNA conjugate and PLL showed a remarkable growth-inhibitory effect (IC_{50} = 6 nM) on the HuH-7 spheroids, inducing long-term apoptotic cell death by means of RecQL1 siRNA. Several important factors are likely to be synergistically involved in the pronounced growth-inhibitory effect of the PEGylated polyplexes, such as the improvement of the stability against enzymatic degradation, smooth penetration into the spheroid interior, and enhancement of the cellular uptake through ASGP receptor-mediated endocytosis. In sharp contrast, the lipoplexes showed almost no growth-inhibitory effect even at a siRNA concentration 100 nM in the HuH-7 spheroids, presumably due to the poor penetration into the spheroids. Furthermore, spheroids derived directly from patients' tumor tissues offer the opportunity of studying the efficacy of delivery systems in the unique tumor cell microenvironments characteristic to individual patients. Thus, MCTSs, as an in vitro tumor model, are expected to be useful in the assessment of the usefulness of siRNA carrier systems in tumor targeting.

Experimental Section

Materials: PLLs (degree of polymerization (DP) = 40, M_w = 8300; DP = 100, M_w = 20900; DP = 460, M_w = 75900) were purchased from Sigma. OligofectAMINE and LipofectAMINE were purchased from Invitrogen. 5′-Thiol-modified sense RNAs (HS-$(CH_2)_6$-CUU ACG CUG AGU ACU UCG AdTdT-3′, firefly luciferase, pGL3-control sense sequence[1] and HS-$(CH_2)_6$-GUU CAG ACC ACU UCA GCU UdTdT-3′, RecQL1, sense sequence[10]) and unmodified antisense RNA (5′-UCG AAG UAC UCA GCG UAA GdTdT-3′, firefly luciferase, pGL3-control antisense sequence and 5′- AAG CUG AAG UGG UCU GAA CdTdT-3′, RecQL1, antisense sequence) were purchased from Dharmacon. Water was purified using a Milli-Q instrument (MILLIPORE). Plasmid DNAs (pDNA) encoding firefly luciferase (pGL3-Control, Promega; 5256 bp) and renilla luciferase (pRL-TK, Promega; 4045 bp) were amplified using EndoFree Plasmid Maxi or Mega Kits (QIAGEN). The DNA concentration was determined by reading the absorbance at 260 nm. A spheroid culture plate, Celltight Spheroid Culture Plate, was purchased from Sumitomo Bakelite. MTT assay reagents and a double staining kit (Live/Dead assay) were purchased from DOJINDO. A Magic Red Caspase Detection Kit was purchased from Immunochemistry Technologies.

Preparation of the PEGylated polyplexes: The PEG-siRNA conjugates were prepared as described in the previous report.[5] Specific amounts of the PEG-siRNA conjugate (50 μM) and PLL were separately added to 10 mM Tris-HCl buffer (pH 7.4) to prepare the 10 μM stock solutions. The solutions were filtered through a 0.1 μm filter to remove the dust. The PEG-siRNA conjugate in 10 mM Tris-HCl buffer (pH 7.4) was mixed with PLL stock solution in 10 mM Tris-HCl buffer (pH 7.4) at an equal unit molar ratio of phosphate groups in the PEG-siRNA conjugate to amino groups in PLL (N/P = 1), followed by the addition of 10 mM Tris-HCl buffer (pH 7.4), adding 0.3 M NaCl to adjust the ionic strength of the solution to the physiological condition (0.15 M NaCl).

Cell culture: HuH-7 human cancer cells, derived from a hepatocarcinoma cell line, were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University. The monolayer-cultured cells and multicellular spheroids were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg mL^{-1} streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

MTT assay: HuH-7 cells were plated in a 96-well plate (10^4 cells/well) to allow them to reach about 50% confluence after 24 h and the medium was then changed to fresh DMEM with 10% FBS (180 μL/well). To each well, appropriate amounts of sample were added in 20 μL aliquot. After 48 h of incubation, fresh medium (100 μL/well) was added, and further incubation was carried out for 48 h. The metabolic activity of each well was determined by an MTT assay. The optical absorbance was measured at 560 nm using a microplate reader and converted to the percentage relative to that for mock cells (buffer-treated cells).

Growth inhibition of the HuH-7 spheroids: Single-cell suspensions were obtained by the trypsinization of monolayer-cultured HuH-7 cells: 45 μL of single-cell suspensions (80 cells) were seeded in individual wells of a 96-well Celltight Spheroid Culture Plate to form the 100 μm HuH-7 spheroids. After 24 h, the PEGylated polyplexes (N/P = 1), siRNA, Lac-PEG-siRNA conjugate or OligofectAMINE/siRNA (5 μL/well) were added to the well at the prescribed concentration on day 0. Fresh DMEM with 10% FBS was added to each wells on day 2 (25 μL/well), 7 (50 μL/well), 10 (50 μL/well), and 14 (50 μL/well), compensating for the decrease in the medium volume due to the natural evaporation. The perpendicular diameters of the HuH-7 spheroids were measured by means of phase-contrast microscope (Olympus IX71) on day 0, 2, 4, 7, 10, 14, and 21. The volume of the HuH-7 spheroids was calculated using the following formula:[21]

$$4\pi a^2 b/3 = \text{Tumor Volume } (\text{mm}^3), \quad (1)$$

where *a* and *b* are the smallest and largest radius (mm) of the HuH-7 spheroids, respectively.

Distribution study of FITC-labeled oligodeoxynucleotide in the HuH-7 spheroids: 5′-FITC-labeled (fluorescein isothiocyanate) oligodeoxynucleotide (ODN) having the same antisense sequence as the firefly luciferase siRNA was hybridized with the sense firefly luciferase PEG-ODN conjugate to form an FITC-labeled Lac-PEG-dsODN conjugate. The lac-PEGylated polyplex was then prepared by mixing FITC-labeled Lac-PEG-dsODN conjugate with PLL (DP = 100). FITC-labeled dsDNA/OligofectAMINE was also prepared as the control. FITC-PEGylated polyplexes or FITC-dsODN/OligofectAMINE were added to the 100 μm HuH-7 spheroids at conjugate or siRNA concentration of 400 nM, and incubated for 48 h. After the incubation, the HuH-7 spheroids were washed three times with phosphate-buffered saline (PBS) and imaged directly in the cell culture medium using a confocal fluorescence-scanning microscope (Olympus IX71 equipped with a confocal IX2-DSU system and an appropriate filter).

Live/Dead staining assay: A Live/Dead staining assay was carried out using a double staining kit. The staining solution (15 μL) containing calcein-acetoxymethyl (10 μM) and propidium iodide (30 μM) were added to the HuH-7 spheroids (100 μm initial diameter) treated with the lac-PEGylated polyplexes (50 nM), OligofectAMINE/siRNA (100 nM), or mock on day 0, 2, 4, 7, 10, 14, and 21. After 2 h of incubation, the HuH-7 spheroids were washed three times with PBS and imaged directly in the cell culture medium using a confocal fluorescence-scanning microscope (Olympus IX71 equipped with a confocal IX2-DSU system and an appropriate filter).

Detection of apoptosis: The detection of apoptosis was carried out using a Magic Red Caspase Detection Kit. Staining solution (5 μL) containing MR-$(DEVD)_2$ (30 μM) were added to the HuH-7 spheroids (100 μm initial diameter) treated with the lac-PEGylated polyplexes (50 nM), OligofectAMINE/siRNA (100 nM) or mock on day 0, 1, 2, 4, 7, 10, 14, and 21. After 2 h of incubation, the HuH-7 spheroids were washed three times with PBS and imaged directly in the cell culture medium using a confocal fluorescence-scanning microscope (Olympus IX71 equipped with a confocal IX2-DSU system and an appropriate filter).

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