Simultaneous Drug and Gene Delivery from the Biodegradable Poly(ε-caprolactone) Nanofibers for the Treatment of Liver Cancer

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In this study, we present anti-cancer drug containing nanofiber-mediated gene delivery to treat liver cancer. Electro-spun nanofibers have big potential for local delivery and sustained release of therapeutic gene and drugs. We reported a temperature-responsive nanofibers mainly compounded by branched poly(ε-caprolactone) (PCL) macro-monomers and anti-cancer drug paclitaxel. The nanofiber could be administrated into liver tumors to dramatically hinder their growth and prevent their metastasis. As a result, paclitaxel encapsulated PCL (PTX/PCL) nanofibers with diameters of around several tens nanometers to 10 nm were successfully obtained by electro-spinning and observed in scanning electron microscopy (SEM). Nanoparticles composed of disulfide cross-linked branched PEI (ssPEI) and anti-cancer therapeutic gene miRNA-145 were complexed based on the electrostatic interaction and coated over the paclitaxel-loaded nanofiber. MicroRNA 145/ssPEI nanoparticles (MSNs) immobilized on the PTX/PCL nanofiber showed time-dependent sustained release of the microRNA for enhanced uptake in neighboring liver cancer cells without any noticeable cytotoxicity. From this study we are expecting a synergistic effect on the cancer cell suppression since we have combined the drug and gene delivery. This approach uses the nanofibers and nanoparticles together for the treatment of cancer and the detailed investigation in vitro and in vivo must be conducted for the practicality of this study. The polymer is biodegradable and the toxicity issues must be cleared by our approach.

Keywords: Gene Delivery, Paclitaxel Encapsulated PCL (PTX/PCL) Nanofiber, MicroRNA 145.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most predominant primary liver cancer, found to be prevalent in certain parts of Asia and sub-Saharan Africa. The recurrence rate after resection in the HCC patient is almost 50–80% at 5 years, resulting from portal venous invasion or imperceptible intrahepatic metastases. The current approach to HCC treatment is combination of chemotherapy and surgical intervention, and the other available regional treatment methods include thermal ablative techniques, ethanol injection, directed radiotherapy, and hepatic arterial infusion chemotherapy. Although the application of these percutaneous or local regional interventions have shown a promising effect in tumor suppression, their successful treatments are severely hindered by some issues, such as technical complexity involved in operation and cost. Nowadays, localized and controlled anti-tumor drug delivery by using biodegradable polymer electrospun nanofiber as drug carriers have shown to be a promising tool for the following two reasons, i.e., (a) improving the local drug availability in tumor area and (b) reducing the non-specific tissue targeting that leads systemic toxicity. Application of nanofiber in liver cancer treatment may offer a
function of homeostasis to the normal neighboring liver tissue, because they have large area of porosity. Poly(β-caprolactone) (PCL) is a flexible polymer, widely used in many biomedical application due to its excellent property like biocompatibility and biodegradation. Cross-linked PCL can control the on-off permeation of the hydrophobic drug due to their reversible crystallizing and melting property. Among the available chemotherapeutic drugs, Paclitaxel (PTX) is used for the various cancer treatments such as breast, ovarian, and liver cancer. Administration of PTX to the cancer patients poses certain side effects and induces the toxicity to normal cells, and therefore a specific PTX delivery system could avoid this limitation. MicroRNAs are double stranded RNA expressed endogenously in cells in order to regulate the expression of protein and maintain the protein homeostasis, but in HCC cells several miRNAs are found to have aberrant expression. Among those, a tumor suppressor miRNA-145 is found to be down-regulated and many studies have also shown that up-regulation of this miRNA significantly inhibit the proliferation and metastasis of HCC tumor. Overall, the current objective of our study is to produce a platform for delivery of both drug and gene specific to the cancer cells. Therefore in this study, we focused on synthesizing a PTX loaded PCL nanofibers using electrospinning technique and nanoparticles containing disulfide cross-linked PEI carrying microRNA-145 are immobilized over PTX loaded nanofiber (Fig. 1). Hence, release of both PTX and miRNA145 from the nanofiber will promote apoptosis in the HCC cells as well as reduction in the tumor invasion.

2. MATERIALS AND METHOD

2.1. Materials

ε-Caprolactone and tetra methylene glycol were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). ε-Caprolactone was purified by distillation over calcium hydride under reduced pressure. Tetrahydrofuran (THF), hexane and other organic solvents used in this work were purchased from Wako Pure Chemical Industries (Osaka, Japan). Poly(D, L-lactide-co-glycolide) was purchased from Sigma-Aldrich (Steinheim, Germany). Milli-Q water (Merck, Millipore, MA, USA) was used in all experiments. Without specific description, all of the chemicals were used as received. Paclitaxel was dissolved in 1 g 2-branched poly(ε-caprolactone) (PCL) was dissolved in HFIP. The electrospinning solutions were prepared by mixing two solutions of PTX/PCL solution and then electrospun from the syringe at a rate of 0.5 mL/h. Surface morphologies of PTX-containing PCL (PTX/PCL) nanofiber were examined by scanning electron microscopy (SEM, Hitachi S-4800, Japan).

2.2. Electro-Spinning of 2-Branched Poly(ε-caprolactone) Macro-Monomers and SEM Observation of Nanofiber Membrane

2-branched poly(ε-caprolactone) macro-monomer was synthesized as previously described. Ten milligrams paclitaxel was dissolved in 1 g 2-branched poly(ε-caprolactone) (PCL) was dissolved in HFIP. The electrospinning solutions were prepared by mixing two solutions of PTX/PCL solution and then electrospun from the syringe at a rate of 0.5 mL/h. Surface morphologies of PTX-containing PCL (PTX/PCL) nanofiber were examined by scanning electron microscopy (SEM, Hitachi S-4800, Japan).

2.3. Measurement of the Molecular Weight and Melting Temperature and Tensile Test

Molecular weight was calculated by 1H NMR and proved by gel permeation chromatography. The melting temperature was measured by differential scanning calorimetry (DSC) (DSC 6100, Seiko Instruments Inc., Chiba, Japan) at 5 °C min⁻¹ of ramping rate. Tensile test was carried out using Shimadzu EZ-S Tabletop Testers.

2.4. Formulation of Immobilization of miR 145/ssPEI Nanoparticles (MSN)

MSN were prepared at an N/P ratio of 10 and treated over the nanofiber at the density of 0.5 and 1 μg/cm² of miR-145 so that the nanoparticles would be immobilized on the PTX/PCL nanofiber surface. MSNs were prepared using phosphate buffered saline (PBS, pH 7.4). ssPEI solution was added drop-wise to solution containing miR-145.
and vortexed for 10 s, followed by 15 min incubation at room temperature. The nanoparticles were then immobilized on PTX/PCL nanofiber surface. PTX/PCL nanofiber was incubated with pre-formed MSN complexes for 24 h and washed twice with PBS.

2.5. Binding Efficiency and Release of MSN on the PTX/PCL Nanofiber

The YOYO1-labeled miR-145 was added to ssPEI and incubated at RT for 30 min in order to form the MSN complex. The formed MSN was immobilized on the nanofiber surface. The immobilization and release of MSNs from the PTX/PCL nanofiber surface was checked by measuring the absorbance of supernatant samples collected at regular intervals using UV-spectrometer.

2.6. In Vitro PTX Release

In vitro release of PTX from the prepared PTX/PCL nanofiber was performed using the dialysis method over 16 days. Five mg of PTX/PCL nanofiber was put into 5 ml in phosphate buffer containing 0.05% of tween-20 in dialysis bag. The resulting suspension was placed into a dialysis bag (MWCO 3,500, Spectrum Laboratories, Inc., CA, USA), sealed, and placed in a beaker containing the release medium (PBS, containing 0.05% (v/v) of tween-20, 1L). Tween-20 was added to increase the solubility of PTX in water. Aliquots with 1 ml each were reserved and replaced with an equal volume of fresh medium every day. The released PTX amounts were quantified by HPLC (LUNA C18 column; Shimadzu, Kyoto, Japan).

2.7. Cell Culture and Transfection

Transfection studies were performed with the HuH-7 hepatocyte derived cellular carcinoma cell line cultured at 37 °C with a 5% CO2 atmosphere in RPMI-1640 medium (HyClone, South Korea) supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (FBS) and 1% HEPES buffer (Sigma, USA). Cells were seeded on and PTX/PCL nanofiber. For reverse transfection, cells were seeded following MSNs immobilization. After transfection of 24 h to 48 h, HuH-7 cells were washed with PBS.

2.8. Cell Viability Assay

MSNs immobilized on the PCL nanofiber surface were incubated at RT for 24 h. After incubation, HuH-7 cells suspended in serum-free medium were seeded at a density of 5 × 10^4 cells/well in 24-well plate and incubated at 37 °C in a humidified atmosphere of 5% CO2. The supernatants were harvested at the 24 h and measured by MTS assay.

2.9. Suppression of HuH-7 Cell Proliferation by MTS Assay

MiR-145/ssPEI and luciferase/ssPEI immobilized on the PCL nanofiber surface were incubated at RT. After

![Figure 2](image-url)
incubation, HuH-7 cells suspended in serum-free medium were seeded at a density of 5 × 10⁴ cells/well in 24-well plate and incubated at 37 °C in a humidified atmosphere of 5% CO₂. The supernatants were harvested at the 24 h, 48 h and measured by MTS assay.

2.10. Measurement of microRNA145-GFP and c-Myc Expression at Protein Level by Western Blot

The HuH-7 cells transfected with miR-145-GFP (0.5, 1 µg) were lysed after 24 h, and protein was extracted using PRO-PREP™ (iNtRON, Korea). For western blot, equal amounts of protein were separated on sodium dodecyl sulfonate (SDS)-PAGE, transferred onto a nitrocellulose membrane, blocked, and incubated for 1 h with anti-GFP and anti-c-Myc antibodies. After washing, the membrane was incubated with a horseradish peroxidase-labeled secondary antibody. The bands were analyzed using a luminescent image analyzer (LAS-3000).

3. RESULTS AND DISCUSSION

3.1. Characterization of PTX/PCL Nanofiber

The random orientation of the nanofiber is clearly shown in the SEM image (Fig. 2(A)). The molecular weight was calculated from GPC and ¹H NMR results and listed in Table I. Figure 2(B) showed DSC thermograms of branched PCL with melting temperature (T_m) at 58.5 °C and result of crystallinity of branched PCL indicated that the PCL diol formed a higher crystallinity of 56.3%. The representative stress–strain curves of fabricated PCL and PTX/PCL nanofibers were shown in Figure 2(C). Although PTX was incorporated inside PCL nanofibers, Young’s modulus of PTX/PCL nanofiber was not influenced as compared with PCL nanofiber.

3.2. The Immobilization of MSN on PTX/PCL Nanofiber Surface

MSNs was immobilized on the nanofiber surface and then measured with UV-spectrometer after labeling with YOYO1 dye. More than 80% of the MSNs were bound over PTX/PCL surface, and the binding efficiency of the MSNs on PTX/PCL nanofiber surface was not much affected by the treated DNA concentration (Fig. 3(A)).

Figure 3. (A) The binding efficiency of YOYO1 labeled MSN on PTX/PCL nanofiber surface. The unbound miR-145 was measured by a UV spectrophotometer. (B) The release kinetics of miR-145 from PTX/PCL nanofiber surface was measured at various time intervals, by UV spectrophotometer from 1 h to 72 h. The release of the MSN from PTX/PCL nanofiber was reached to be 78% at 3-day post-incubation. (C) in vitro drug release profiles from PTX/PCL nanofiber over 16 days. Data are expressed as mean±S.D. (n = 3).

Figure 4. The cell viability of HuH-7 cultured on luciferase plasmid/ssPEI nanoparticles-immobilized PTX/PCL nanofiber surface. Different amounts of luciferase plasmid (0.5 and 1 µg) were complexed with ssPEI at a fixed N/P ratio of 10 and immobilized on the PTX/PCL nanofiber surface. The cellular viability of HuH on the surface was measured using MTS assay.

Figure 5. Cell proliferation for miR-145 and p-Luc with the ssPEI and bPEI carrier was tested in HuH-7 cells from 24 h. Mir 145 and p-Luc concentration from 1 µg.
3.3. Release of MSN from PTX/PCL Nanofiber
The release of MSNs from the PTX/PCL nanofiber surface in PBS buffer was analyzed by measuring the absorbance of the supernatants; from the absorbance value, the percentage of MSNs released from the PTX/PCL nanofiber was calculated. At 72 h, 70% of the MSN was released (Fig. 3(B)).

3.4. In Vitro PTX Release
The in vitro cumulative release profiles of PTX from PTX/PCL nanofiber are shown in Figure 3(C). The amount of drug released from the PCL nanofiber was reached up to 10% at 1-day incubation based on cumulative release curve. At 16-day post-incubation, the PTX was released to approximately 20%. These results indicate slower and continuous release may be attributed to the diffusion of the drug localized in PCL nanofiber.

3.5. Cytotoxicity Assay
The luciferase plasmid was complexed with ssPEI to test their cytotoxicity against HuH-7 cells. Varying concentrations of luciferase, 0.5 μg and 1 μg, were tested for viability. The luciferase plasmid/ssPEI nanoparticles showed cell viability higher than 90% (Fig. 4).

3.6. Suppression of HuH-7 Cell Proliferation by MTS Assay
MSNs immobilized on the PTX/PCL nanofiber surface were incubated at RT. After incubation, HuH-7 cells were suspended on the MSN-PTX/PCL nanofiber surface. The supernatants were harvested at the 24 h, 48 h and measured by MTS assay. The results showed that at 48 h, MSN-PTX/PCL nanofiber showed more cell growth suppression compared to PTX/PCL nanofiber (Fig. 5).

3.7. GFP and c-Myc Protein Expression in HuH-7 Cells Mediated by miR-145 Nanoparticles
HuH-7 cells were seeded on the miR-145 nanoparticles-immobilized PTX/PCL nanofiber. GFP and microRNA 145 downstream pathway protein c-Myc expression in HuH-7 cells was confirmed by Western blot data as shown in Figure 5, which indicated that therapeutic miR-145 was also expressed in the treated cells (Fig. 6).

4. CONCLUSION
We studied microRNA/ssPEI nanoparticles immobilized on the PTX/PCL nanofiber for the treatment of liver cancer. The electrospun nanofiber allowed uniform distribution of PTX on the PCL nanofiber, sustained release of PTX and miR-145 for in vitro tumor growth inhibition. From this study a synergistic effect on the cancer cell suppression was expected by the concomitant delivery of drug and gene.

Acknowledgment: This work was financially supported by the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A120899 and HI14C0187); the Leading Foreign Research Institute Recruitment Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (MEST) (2011-0030034, NRF-2013R1A2A2A01004668 and 2013K2A2A4000604); and the Pioneer Research Center Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future Planning (2014M3C1A3053035). IKP acknowledges the support from a grant (CR14073-3) of the Chonnam National University Hospital Research Institute of Clinical Medicine.

References and Notes

Received: 30 November 2014. Accepted: 27 January 2015.