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ORIGINAL RESEARCH

In Vitro PDGF-B Gene Silencing Studies and *In Vivo* Delivery of siRNA to the Rat Kidney Using Chitosan/siRNA Nanoplexes

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ABSTRACT

The targeting of specific genes responsible from onset and progression of kidney diseases offer a new therapeutic strategy in the field of renal gene therapy. The altered expression of platelet derived growth factor (PDGF) is an important marker of renal diseases. In this study, we investigated *in vitro* gene silencing efficiency of chitosan nanoplexes containing PDGF-B and PDGFR-β targeted siRNAs in the kidney cell lines including HEK-293 and MDCK and delivery to the kidney as an *in vivo* delivery system. As a result, PDGF-B expression was significantly inhibited by co-delivery of chitosan/siPDGF-B+siPDGFR-β nanoplexes prepared using in the different weight ratios (10/1, 20/1 and 50/1). When 20/1 and 50/1 weight ratios of chitosan nanoplexes were i.v. injected to rats, chitosan/FITC-siPDGFB nanoplexes were reached to kidney tissue at 4 h after intravenous injection. These results suggest that delivery of siRNA using chitosan nanoplexes may be effective for the therapy of kidney diseases.

Keywords: siRNA, PDGF-B, PDGFR-β, chitosan, nanoplexes, kidney

INTRODUCTION

Kidney diseases are an important health problem in worldwide. Diabetic and non-diabetic kidney diseases including glomerular, vascular, tubulointerstitial, cystic diseases are cause of kidney diseases (1). Classic pharmacologic approaches have been widely used for treatment of kidney diseases. Recently, the targeting of specific genes responsible from onset and progression of kidney diseases offer a new therapeutic strategy in the field of renal gene therapy (2). One of these strategies involves targeting mRNA by using small interfering RNAs (siRNAs), which induce sequence-specific gene silencing. Several studies reported that the direct delivery of naked siRNA has sufficient effect in occurring of RNA interference (RNAi) mechanism (3). However, if siRNA is not chemically modified or use as a free form without delivery system, it has little chance in the *in vivo* applications. The most important limitation of siRNAs is that they have poor pharmacokinetic profile due to easily degraded by nucleases. siRNAs have short half-life in serum. siRNAs are

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small size in the 21-23 nucleotide double-stranded structure. When administered as systemically, they are rapidly excreted through urine. Thus, the rapid clearance by kidneys reduces half-life of siRNA (4). Although this situation is undesired effect, kidney diseases may provide an important target for siRNA therapy (2). One of the other major difficulties for siRNA therapy is not freely cross the cell membrane due to its large molecular weight (~13 kDa) and polyanionic nature (~40 negative phosphate charges) (5). Therefore, efficient delivery systems in the *in vitro* and *in vivo* conditions are prerequest for effective gene silencing by siRNA in target cells.

The delivery of siRNAs to the kidney can be lead to better therapeutic outcomes because of reducing repeated administrations or dose and minimizing side effects and toxicity (6). Chitosan is a cationic biopolymer that has many advantages in terms of specific delivery, cellular uptake and intracellular trafficking to improve the therapeutic effect of siRNA (7). The many studies using chitosan as delivery system for siRNA have been made in our laboratory. Previously, we reported that chitosan is an efficient delivery system for nucleic acid-based drugs (8, 9).

There are several reports related with targeting to the kidney with nanoparticle based therapeutics. Choi *et al.* showed that PEGylated gold nanoparticles (AuNPs) in the under of 100 nm particle size can be targeted the mesangium of the kidney (10). Thus, they have highlighted importance for the therapy of kidney diseases of the accumulation of PEGylated Au NPs in the kidneys.

Zuckerman *et al.* reported efficient delivery of siRNA to the glomerular mesangium by intravenously administration of polycationic cyclodextrin nanoparticles containing siRNA. They showed suppression of enhanced green fluorescent protein (EGFP) in the mesangial cells following intravenous administration of nanoparticles (11).

Gao *et al.* showed to target siRNA specifically to the proximal tubule epithelial cells (PTECs) in the kidneys in mice when administered intravenously of chitosan/siRNA nanoparticles to the megalin gene knockout mice. They have reported chitosan nanoparticles are potential carrier for knockdown of specific genes in PTECs. (12).

Platelet derived growth factor (PDGF) is one of the best characterized growth factors in kidney diseases. PDGFs are important autocrine and paracrine mitogens and survival factors in many cells of mesenchymal origin (13). PDGF-B is a ligand for PDGF receptors α and β (PDGFR-α and PDGFR-β). The abnormal expressions of PDGF-B and PDGFRs are lead to development of glomerular diseases in human and experimental models (13). The targeted therapeutic strategies to PDGFs offer promising approaches to kidney diseases (6). Here, we investigated *in vitro* PDGF-B gene silencing efficiency of prepared chitosan nanoplexes with siRNAs targeting not only PDGF-B but also its receptor PDGFR-β in the kidney cell lines and also targeting to the kidney of chitosan nanoplexes as *in vivo* delivery system.

MATERIALS AND METHODS

Chemicals

siPDGF-B and siPDGFR-β were obtained from Qiagen (USA). FITC-labelled siPDGF-B was obtained from Dharmacon (USA). Chitosan (75 kDa; 75%–85% deacetylation) was purchased from Sigma (St. Louis, MO, U.S.A.). All the substances used in this study were of molecular grade.

The Control and Preparation of Chitosan/siRNA Nanoplexes

Chitosan/siRNA nanoplexes were formed by electrostatic interactions between cationic chitosan polymer and anionic siRNA molecule. Low molecular weight chitosan stock solution (2.5 mg/ml) was prepared in 1% acetic acid solution and filtered through 0.45 μm membrane filter. Chitosan/siRNA nanoplexes were prepared in different weight ratios (10/1, 20/1 and 50/1) by adding into chitosan solutions in different amounts of siRNA solutions in the constant amount. The mixed solution was vortexed and incubated for 30 min at room temperature for forming nanoplexes completely. The formation of nanoplexes was checked by agarose gel electrophoresis using 2% gel at 200V/80 mA (14).

Particle size and zeta potential values of chitosan/siRNA nanoplexes in PBS were measured by photon correlation spectrosccopy using a Zetasizer NanoZS (Malvern Instruments, NanoZS ZEN 3600, UK) after dispersed in PBS (pH 7.4). All measurements were carried out in triplicate with a temperature equilibration time of 1 minute at 25°C.

In vitro Transfection and Gene Silencing Studies

Human Embryonic Kidney 293 (HEK293) and Madin-Darby Canine Kidney (MDCK) cells, the commonly used kidney cell lines, were obtained from the American Type Culture Collection. These cells were utilized because they constitutively express PDGF in the presence of 20% fetal bovine serum (FBS) in medium (15). Cells maintained in Dulbecco's modified Eagle medium (DMEM)

supplemented with 20% FBS and 100 IU/ml penicillin and 50 mg/ml streptomycin in a humidified in incubator 5% CO_2 atmosphere at 37°C and were passaged each three days. Cells were plated in 24 well plates at a cell density of 5 x10^5 cells/well and incubated overnight. Transfections were performed on cells that were approximately 70% confluent. Chitosan/siPDGF-B and siPDGFR-β nanoplexes in the different weight ratios (10/1, 20/1 and 50/1) were added to cells and incubated for 48 and 72 hours for transfection.

PDGF-B Quantification by ELISA

ELISA for the detection of PDGF-B protein expression levels in cell culture supernatants were performed according to the manufacturer's instructions using a sandwich ELISA (USCN Life, China), with absorbance being determined by spectrophotometry at 450 nm. Calibration curve was generated using this standard, at concentrations between 0 and 500 pg/mL. All experiments were repeated thwice and data were expressed as mean SEM.

***In vivo* Study of Chitosan/siFITC-labeled PDGF-B Nanoplexes to the Kidney**

In vivo targeting study to the kidney of chitosan/siRNA nanoplexes, 20 µg FITC labelled PDGF-B siRNA was formulated with chitosan in 20/1 and 50/1 weight ratios. Chitosan/FITC-siPDGF-B nanoplexes were injected into tail vein of Sprague-Dawley rats. Rats were sacrificed 4 hours after the injection and kidneys were collected and snap frozen in liquid nitrogen. The frozen tissue was used for cryo-sections. Sections were examined by fluorescence microscopy. All procedures of animal work were approved by Animal Ethic Committee of Inonu University (2012/A-76).

Statistical Analysis

The results are expressed as the mean ± standard deviation. Statistical analysis of differences between groups was performed using an unpaired Student's t-test by SPSS 16.0. P values less than 0.05 were considered statistically significant.

RESULTS and DISCUSSION

Characterization of Nanoplexes

siRNAs have anionic phosphodiester backbones with negative charge ratios and therefore they may interact electrostatically with cationic chitosan polymer to form chitosan nanoplexes (5, 16). After preparation, the formation of chitosan/siPDGF-B+siPDGFR-β nanoplexes were checked by agarose gel electrophoresis. No free siRNA were detected on the gel (Fig.1).

Figure 1. Agarose gel analysis of the chitosan/siPDGF-B+siPDGFR-β nanoplexes in the different weight ratios (1. Free siRNA, 2-5. 10/1, 20/1, 30/1, 50/1).

The physicochemical properties of the nanoplexes are the major factor of the gene silencing efficiency (17). The mean diameters and zeta potentials of all nanoplexes were analyzed (Table 1). The zeta potential values of chitosan/siPDGF-B nanoplexes (10/1-50/1) were ranged from +10.1 to +28.7 mV and their particle sizes were ranged from 262 to 514 nm. The zeta potential values of chitosan/siPDGF-B+siPDGFR-β nanoplexes (10/1-50/1) were ranged from +7.8 to +22.3 mV and their particle sizes were ranged from 261 to 341 nm. The zeta potential and particle size values of nanoplexes increased with increasing amount of chitosan. Numerous studies have reported that particle size is an important parameter for cellular uptake in targeted drug delivery (7, 10, 16). When the particles have average diameter between 200 and 500 nm, they can be introduced into target cells by endocytosis and subsequently permeate the nuclear membranes through the nuclear pores (18, 19).

Table 1. Particle size and zeta potential measurements of chitosan/siRNA nanoplexes.

Formulations	Zeta Potential (mV±SD)	Particle Size (nm±SD)
Free siPDGF-B	- 13,2 ± 3,2	
Chitosan	35,0 ± 2,8	
Chitosan/siPDGF-B 10/1	10,1 ± 0,1	262 ± 21,0
Chitosan/siPDGF-B 20/1	15,0 ± 0,6	333 ± 2,4
Chitosan/siPDGF-B 50/1	28,7 ± 0,7	514 ± 13,8
Chitosan/siPDGF-B+siPDGFR-β 10/1	7,8 ± 2,8	261 ± 2,1
Chitosan/siPDGF-B+siPDGFR-β 20/1	10,6 ± 1,5	307 ± 3,9
Chitosan/siPDGF-B+siPDGFR-β 50/1	22,3 ± 6,9	341 ± 5,8

The Suppression of PDGF-B Expression in Kidney Cells

PDGF is one of the best described growth factors in kidney diseases. Changed expression levels of PDGF and its receptors lead to many renal diseases. Therefore, specific anti-PDGF therapy approaches have been developed for prevention of renal diseases (13). By the aim of this, we tried to knockdown PDGF-B expression level with chitosan/siPDGF-B+siPDGFR-β nanoplexes in the kidney epithelial cells.

In vitro gene silencing study, we firstly investigated endogenous PDGF-B expression in the MDCK and HEK293 cells. Endogenous PDGF-B expression was measured as 214 pg/ml in HEK293 cells and 187 pg/ml in MDCK cells. Then, to investigate the PDGF-B gene silencing effect of chitosan/siPDGF-B, chitosan/siPDGFR-β and chitosan/siPDGF-B+siPDGFR-β nanoplexes in both cell lines, nanoplexes in the different weight ratios (10/1-50/1) were transfected to cells and incubated 48 h (Figs. 2-5). After transfection, PDGF-B expression was decreased as depend on weight ratio of nanoplexes and co-delivery of siPDGF-B and siPDGFR-β in the both cells. As shown in Figs. 2-5, the co-delivery of siPDGF-B and siPDGFR-β provided high PDGF-B downregulation effect, comparing to single formulations. Moreover, the highest PDGF-B downregulation effect was found in the HEK293 and MDCK cells treated with prepared 50/1 weight ratio nanoplexes, respectively 71% and 57%. Gao et al. transfected chitosan/siRNA nanoparticles containing 50 nM siRNA at N/P=60 and N/P=10 ratio to the EGFP expressing H1299 cell line (12). *In vitro* transfection results showed EGFP knockdown by N/P ratio of 60 was higher than that of 10. These results were similar to our results. They were investigated silencing of gene expression in MDCK cells of chitosan nanoparticles containing siRNA targeted aquaporin 1 (AQP1), which is predominantly expressed in PTECs. Chitosan nanoparticles containing two of three siRNA sequences designed to target mouse AQP1 showed a strong knockdown mRNA and protein expression. From the above results, it was suggested that co-formulation of siRNAs targeting growth factor and its receptor would promote gene silencing efficiency.

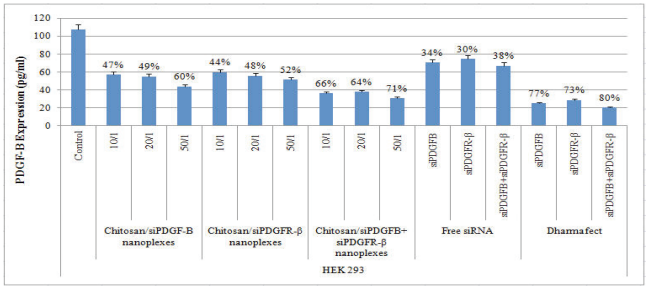

Figure 2. PDGF-B gene silencing efficiency of the chitosan/siPDGF-B+siPDGFR-β nanoplexes which was determined by ELISA in HEK 293 cells.

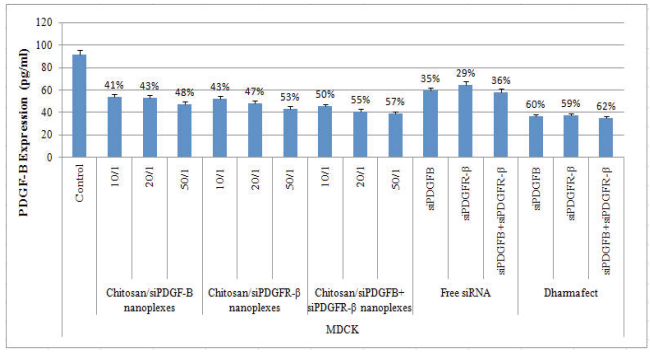

Figure 3. PDGF-B gene silencing efficiency of the chitosan/siPDGF-B+siPDGFR-β nanoplexes which was determined by ELISA in MDCK cells.

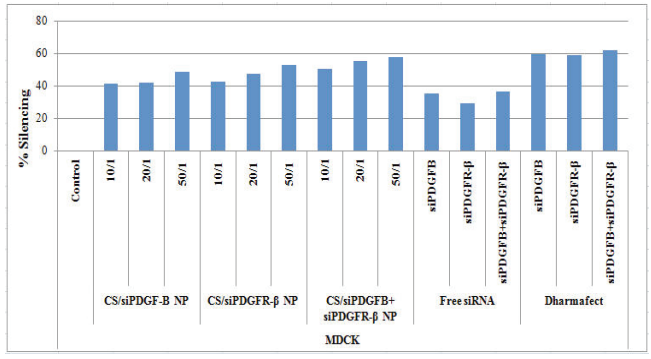

Figure 4. Percent PDGF-B gene silencing in the HEK293 cell line.

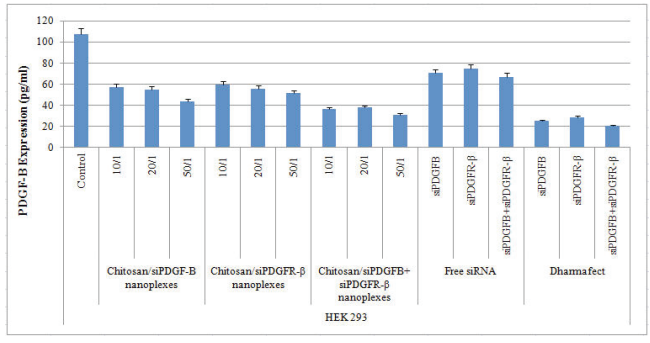

Figure 5. Percent PDGF-B gene silencing in the MDCK cell line.

In Vivo Delivery of Chitosan/FITC-siPDGF-B Nanoplexes to Kidney

To test whether or not reaching or accumulating to the kidney of chitosan/siRNA nanoplexes, chitosan/FITC-labelled siPDGF-B nanoplexes were systematically administered to rats formulated with chitosan in 20/1 and 50/1 weight ratios. We examined fluorescence intensity of the kidneys by fluorescence microscopy analysis after at 4 hours

of transfection. As shown in Figure 6, strong fluorescence intensity was shown in kidney tissue at 4 h after intravenous injection of chitosan/FITC-siPDGF-B nanoplexes. In terms of the fluorescence intensity between 20/1 and 50/1 weight ratios was not seen any difference. A lower fluorescence intensity in kidney was found at 4 h after intravenous injection of free FITC-siPDGF-B. Gao *et al.* reported that when chitosan/Cy5-labeled siRNA nanoparticles were imaged by fluorescence living imaging system, Cy5 signals were apparent in the kidney after 20-30 min and peaked at 3-4 hrs, with some signal remaining more than 48 hrs post injection (12). Naked siRNA peaked at an earlier time point (30 min) and disappeared almost 1 hr to 3 hrs post injection. These results are in agreement with our results obtained by fluorescence microscopy. Shimizu *et al.* developed to target delivery to kidney mesangium of mitogen-activated protein kinase 1 (MAPK1) siRNA containing poly (ethylene glycol)-poly (L-lysine) nanoparticles (20). They showed strong fluorescence in both kidneys at 3.5 hrs after peritoneal injection of Cy5-labeled siRNA nanoparticles and detected siRNAs in the blood circulation for a prolonged time. Gao et al. suggested that chitosan/siRNA complexes accumulated in the kidneys after intraperitoneal administration and siRNA level in the kidney remained very high even after 24 hrs (21). Chitosan prolonged the blood circulation time of siRNA. Salva *et al.* showed the biodistribution of chitosan/siVEGF complexes to many tissues including kidney, liver, brain, spleen, lungs and muscle. Especially, chitosan/siVEGF complexes were reached to breast tumour within 15 min post injection (22).

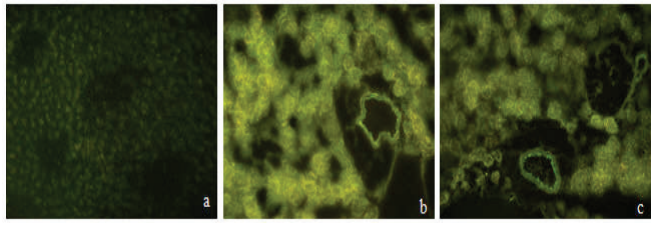

Figure 6. Fluorescence microscopy images of rat kidney at the 4 hours after i.v. administration of free siRNA (**a**) and chitosan/FITC-siPDGF-B nanoplexes in the 20/1 (**b**) and 50/1 (**c**) weight ratios. Thin, thick arrows and arrow head were shown tubules, blood vessels, glomeruli, respectively.

CONCLUSION

In conclusion, chitosan/siRNA nanoplexes may help to clarification of molecular mechanisms and therapy of kidney diseases. Our results suggested that the co-delivery of platelet derived growth factor and its receptor by chitosan nanoplexes would be a promising approach for enhance of gene silencing efficiency.

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In vitro PDGF-B gen susturma çalışmaları ve siRNA'nın rat böbreğine kitosan/siRNA nanopleksleri kullanılarak _in vivo_ taşınması

ÖZ

Böbrek hastalıklarının başlaması ve ilerlemesinden sorumlu olan spesifik genlerin hedeflenmesi renal gen tedavisi alanında yeni bir terapötik stratejidir. Platelet-türevi büyüme faktörünün (PDGF) ekspresyonunun değişmesi böbrek hastalıklarının önemli belirteçlerinden biridir. Bu çalışmada, PDGF-B ve PDGFR-β hedefli siRNA içeren kitozan nanoplekslerin HEK-293 ve MDCK hücre hatlarında *in vitro* susturma etkinlikleri ve *in vivo* olarak böbreğe taşınması araştırılmıştır. Sonuç olarak, farklı oranlarda (10/1, 20/1 ve 50/1) hazırlanan kitozan/siPDGF-B+siPDGFR-β nanoplekslerinin PDGF-B ekspresyonunu anlamlı olarak inhibe ettiği görülmüştür. 20/1 ve 50/1 oranında hazırlanan kitozan nanoplekslerin sıçanlara i.v. enjeksiyonundan 4 saat sonra FITC-siPDGF-B nanoplesklerin böbrek dokusuna ulaştığı gözlenmiştir. Bu sonuçlardan yola çıkılarak, böbrek hastalıklarının tedavisinde siRNA taşınması için kitozan nanoplekslerin etkili olabileceği görülmüştür.

Anahtar kelimeler: siRNA, PDGF-B, PDGFR-β, kitozan, nanopleks, böbrek

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