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Device-based local delivery of siRNA against mammalian target of rapamycin (mTOR) in a murine subcutaneous implant model to inhibit fibrous encapsulation ☆

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ABSTRACT

Fibrous encapsulation of surgically implanted devices is associated with elevated proliferation and activation of fibroblasts in tissues surrounding these implants, frequently causing foreign body complications. Here we test the hypothesis that inhibition of the expression of mammalian target of rapamycin (mTOR) in fibroblasts can mitigate the soft tissue implant foreign body response by suppressing fibrotic responses around implants. In this study, mTOR was knocked down using small interfering RNA (siRNA) conjugated with branched polyethylenimine (bPEI) in fibroblastic lineage cells in serum-based cell culture as shown by both gene and protein analysis. This mTOR knock-down led to an inhibition in fibroblast proliferation by 70% and simultaneous down-regulation in the expression of type I collagen in fibroblasts *in vitro*. These siRNA/bPEI complexes were released from poly(ethylene glycol) (PEG)-based hydrogel coatings surrounding model polymer implants in a subcutaneous rodent model *in vivo*. No significant reduction in fibrous capsule thickness and mTOR expression in the foreign body capsules were observed. The siRNA inefficacy in this *in vivo* implant model was attributed to siRNA dosing limitations in the gel delivery system, and lack of targeting ability of the siRNA complex specifically to fibroblasts. While *in vitro* data supported mTOR knock-down in fibroblast cultures, *in vivo* siRNA delivery must be further improved to produce clinically relevant effects on fibrotic encapsulation around implants.

1. Introduction

The foreign body reaction (FBR) at the tissue/material interface commonly contributes to abnormal inflammation, wound healing responses and tissue fibrosis without effective mitigation [1,2]. In general, monocytes/macrophages are activated at implant surfaces and modulate local host fibroblast function, contributing to often-excessive deposition of collagen matrix around implanted materials (fibrotic capsule), a component of the FBR [1,3]. Recent work [4] demonstrated that macrophage fusion observed around implants alone does not necessarily produce fibrotic encapsulation at the implant sites. Instead, an alternative hypothesis is that fibroproliferation is regulated by growth factors secreted by activated macrophages [3,5,6]. Fibrogenesis induced by implants is characterized by macrophage activation and associated elevated proliferation and activation of fibroblasts that up-regulate collagen production. Therefore, control of inflammation around implants by locally released drugs to reduce cell activation and limit collagen encapsulation of implanted biomaterials has been reported [7–9].

Mammalian target of rapamycin (mTOR) plays a critical role in cell cycle regulation. Rapamycin, a known inhibitor for mTOR [10], can inactivate mTOR specifically. Because mTOR regulates cell proliferation, it has been extensively investigated as a potent target for both anti-cancer [11] and anti-restenotic [12] therapies. Inhibition of mTOR in fibroblasts influences not only proliferation but also collagen production [13,14]. Rapamycin and its analogues are reported to effectively prevent cardiac and pulmonary fibrosis *in vivo* [15,16]. These previous reports describing modulation of mTOR in fibroblasts indicate that mTOR could also be a potent target to prevent implant-induced fibrosis in the context of the FBR.

RNA interference (RNAi) is a powerful tool to knock down specific mRNA expression levels by exploiting a natural intracellular regulatory phenomenon in mammalian species [17–19]. Gene silencing using short interfering RNAs (siRNAs) has many potential therapeutic applications [20]. However, RNAi technology has not yet been clinically useful due to challenges in dosing and effective targeted siRNA delivery systems. Local or topical siRNA therapeutics have been most actively investigated and successful delivery approaches include ocular delivery, respiratory

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delivery, CNS delivery, skin delivery and vaginal delivery where local delivery accesses cell target populations directly [21–25]. One unexplored and promising delivery route is via combination implantable devices for local drug delivery [26]. We therefore demonstrate device-based local delivery of siRNA, testing the hypothesis that delivery of siRNA targeting mTOR (mTOR siRNA) from poly(ethylene glycol) (PEG)-based hydrogel-coated biomaterials can suppress collagen encapsulation elicited from a soft tissue implant-induced FBR.

2. Materials and methods

2.1. Chemicals

Branched polyethylenimine (bPEI) (mol. wt.: 25,000) and dithiothreitol (DTT) were obtained from Sigma-Aldrich (USA). Poly (ethylene glycol) dimethacrylate (PEGDM; mol. wt.: 7500) was synthesized as reported previously [27]. RNase-free water was prepared using diethyl pyrocarbonate (DEPC) (Sigma-Aldrich). All siRNA molecules were purchased from Dharmacon (CO, USA).

2.2. Preparation of siRNA/bPEI complexes

To prepare siRNA/bPEI complexes at various anion/cation charge (NP) ratios, 2 μl of 10 μM mTOR siRNA aqueous solution (sense: GCG GAU GGC UCC UGA CUA UUU, antisense: AUA GUC AGG AGC CAU CCG CUU) was mixed with 2 μl of bPEI solutions of different concentrations (0.016–0.64 μg). The complex mixed solutions were kept at room temperature for 20 min. Then 4 μl of each mixture was electrophoresed using ethidium bromide-stained TBE-based 2% agarose gels run at 80 V for 20 min, followed by visualization with UV light to assess the siRNA/bPEI complex formation.

2.3. Cell culture and siRNA transfection in vitro

Murine NIH 3T3 fibroblasts (American Type Culture Collection, ATCC) were plated at 3×10^4 cells/well in a 12-well plate in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone®, USA) and 1% penicillin–streptomycin (GIBCO), defined for all cell cultures as "complete media", at 37 °C with 5% CO_2 overnight. Cell transfections with siRNA/bPEI complexes at fixed NP ratios in complete media were performed subsequently. siRNA/bPEI complexes for each well are prepared by mixing 7 ul of 20 μM siRNA aqueous solution with 4.48 μl, 2.24 μl, 1.12 μl and 0 μl (NP 20, 10, 5 and 0) of 1 mg/ml bPEI, respectively, in a total volume of 18 μl with RNase-free water. After incubation at room temperature for 20 min, complete media was added to achieve the final volume of 1 ml, yielding a final concentration of siRNA in each well of 140 nM. siRNA transfections were always performed in complete media. After 24-hour incubation at 37 °C under 5% CO_2, culture media was refreshed with 1 ml complete media and the transfected cells were further incubated.

2.4. Cell cytotoxicity and proliferation

3T3 murine fibroblasts were seeded at 3×10^3 cells/well in 96-well plates in complete media. After overnight incubation, cells were transfected with mTOR siRNA/bPEI complexes at different NP ratios (1, 2, 5, 10, 20, and 40 prepared as described above) in complete media, maintaining siRNA concentration at 140 nM. Cytotoxicity of the siRNA/bPEI complexes was determined at 24 h after initial transfection using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA). Media for each well was replaced with 100 μl fresh complete media containing 20 μl of Cell Titer 96 Aqueous One Solution including three wells without cells for background subtraction. Cells were then incubated at 37 °C for 2 h and optical absorbance at 490 nm was then determined using a plate reader (TECAN GENIOS Plus).

To evaluate mTOR siRNA effects on cell proliferation, cells were plated at 3×10^4 cells/well in 6-well plates and transfected with mTOR siRNA/bPEI complexes at an NP ratio of 20 in complete media. Non-targeting siRNA/bPEI complexes with the same NP ratio were used as control. Cultures were refreshed with complete media 24 h later. After incubation at 37 °C for 5 days, relative numbers of cells in each well were determined using the Cell Proliferation Assay (Promega, USA). CellTiter 96 solution-containing media was transferred to 96-well plates for optical reading at 490 nm. In addition, cultured cells were imaged at Day 5 with phase contrast microscopy prior to this assay.

2.5. Western immunoblotting

Cells were lysed by using M-PER Mammalian Protein Extraction reagent (Pierce, USA) with 1X Halt™ protease inhibitor cocktail (Pierce). Insoluble material was removed by centrifuging at 15,000 rpm at 4 °C for 5 min after 20 min on ice. Protein concentrations were measured with the Bio-Rad protein assay system (Bio-Rad, USA). Heat-denatured protein samples (8 μg) were separated on 4–12% SDS-polyacrylamide gels (Invitrogen) and blotted on to cellulose membranes (Bio-Rad). After blocking with bovine serum albumin (BSA) in phosphate buffered saline containing 0.5% Tween 20 (PBST) for 1 h at RT, the filter was incubated overnight with antibody against murine mTOR (2983, Cell Signaling) in 5% BSA/PBST with constant shaking. After three washes with PBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (SA1-200, Affinity BioReagents). Housekeeping controls were detected with an antibody against mouse cyclophilin B (PA1-027, Affinity BioReagents) and HRP-conjugated anti-rabbit IgG. Chemiluminescence was produced with western blotting luminol reagent (Santa Cruz Biotechnology) and gel images captured using a Molecular Imager Gel Doc XR System (Bio-Rad).

2.6. Reverse transcript polymerase chain reaction (RT-PCR)

Total RNA harvests from transfected cells were isolated 48 h after siRNA transfection using an RNeasy Mini Kit (Qiagen). Up to 0.5 μg of RNA was converted to cDNA with the SuperScript III 1st strand RT kit for PCR (Invitrogen). PCR primers were designed for mTOR (forward: 5′-AGC GTA TTG TTG AGG ACT GGC AGA-3′, reverse: 5′-ATC CTG GAG GTT GTT GCC TCT TGA-3′), cyclophilin B (housekeeping control, forward: 5′-GCA ATG GCA AAG GGT TTC TCC ACT-3′, reverse: 5′-AGC GCT TCC CAG ATG AGA ACT TCA-3′), and collagen type 1 alpha 1 (COL1A1) (forward: 5′-AAG AAT GGC GAT CGT GGT GAG ACT-3′, reverse: 5′-TTG AGT CCG TCT TTG CCA GGA GAA-3′) using Primerquest software from Integrated DNA Technologies (IDT, USA). PCR was performed with iTaq DNA polymerase (Bio-Rad), 1.5 mM magnesium chloride, 200 μM each of dNTPs, 500 nM of each primer, and 2 μl of the cDNA. PCR reaction for mTOR was 95 °C for 3 min, followed by 25 cycles with 95 °C for 30 s, 63.9 °C for 30 s, and finally at 72 °C for 1 min. PCR reactions for cyclophilin B and COL1A1 were 95 °C for 3 min, followed by 30 cycles with 95 °C for 30 s, 60 °C for 30 s, and finally at 72 °C for 1 min. PCR products were collected for all three genes and electrophoresed using ethidium bromide-stained TBE-based 2% agarose gels run at 100 V for 30 min.

2.7. Preparation of PEG-based hydrogel release matrix and in vitro controlled release of siRNA/bPEI complexes to cultured cells

Crosslinked hydrogels releasing siRNA were prepared using PEGDM and DTT at a 1:1 stoichiometric ratio of thiols to acrylates by Michael-type addition reactions [28,29]. FITC-labeled siRNA (FITC-siRNA, siGLO® Green, Dharmacon) was used for determining siRNA release kinetics from the PEG-based hydrogels. To encapsulate siRNA in the hydrogels, 2 μg of siRNA and 4.77 μl of 1 mg/ml bPEI (NP = 20) were mixed first and incubated at room temperature for 15 min. Volumes of stock solutions equal to either 4.25 or 8.5 mg of PEGDM and 0.09 or 0.18 mg of