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Local therapeutic efficacy with reduced systemic side effects by rapamycin-loaded subcapsular microspheres

Lucas L. Falke [a, 1], Stefan H. van Vuuren [a, 1], Filis Kazazi-Hyseni [b], Farshad Ramazani [b], Tri Q. Nguyen [a], Gert J. Veldhuis [c], Erik M. Maarseveen [d], Jurjen Zandstra [e], Johan Zuidema [f], Luisa F. Duque [f], Rob Steendam [f], Eliane R. Popa [e], Robbert Jan Kok [b], Roel Goldschmeding [a, *]

[a] Department of Pathology, University Medical Centre Utrecht, The Netherlands
[b] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, The Netherlands
[c] Nanomi B.V., Oldenzaal, The Netherlands
[d] Department of Clinical Pharmacy, University Medical Centre Utrecht, The Netherlands
[e] Department of Pathology and Laboratory Medicine, University Medical Centre Groningen, University of Groningen, The Netherlands
[f] InnoCore Pharmaceuticals, Groningen, The Netherlands

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ABSTRACT

Kidney injury triggers fibrosis, the final common pathway of chronic kidney disease (CKD). The increase of CKD prevalence worldwide urgently calls for new therapies. Available systemic treatment such as rapamycin are associated with serious side effects. To study the potential of local antifibrotic therapy, we administered rapamycin-loaded microspheres under the kidney capsule of ureter-obstructed rats and assessed the local antifibrotic effects and systemic side effects of rapamycin. After 7 days, microsphere depots were easily identifiable under the kidney capsule. Both systemic and local rapamycin treatment reduced intrarenal mTOR activity, myofibroblast accumulation, expression of fibrotic genes, and T-lymphocyte infiltration. Upon local treatment, inhibition of mTOR activity and reduction of myofibroblast accumulation were limited to the immediate vicinity of the subcapsular pocket, while reduction of T-cell infiltration was widespread. In contrast to systemically administered rapamycin, local treatment did not induce off target effects such as weight loss. Thus subcapsular delivery of rapamycin-loaded microspheres successfully inhibited local fibrotic response in UUO with less systemic effects. Therapeutic effect of released rapamycin was most prominent in close vicinity to the implanted microspheres.

1. Introduction

Kidney injury triggers inflammation, irrespective of insult type. The inflammatory response encompasses vascular activation [1], infiltration of inflammatory cells into the renal interstitium [2,3] and production of pro-inflammatory cytokines [1,4,5]. Inadequate resolution of acute renal inflammation and progression to chronic inflammation set the stage for the development of fibrosis, the final common pathway to chronic kidney disease (CKD). Worldwide rapid increase of CKD incidence urgently calls for new therapies.

Evidence exists that the mTOR pathway plays an important role in the mechanisms underlying the progression of CKD. In all eukaryotic organisms mTOR is present and functions as an intracellular nutrient sensor that controls protein synthesis, cell growth and metabolism [6]. There are two mTOR complexes (mTORC1 and mTORC2). The well-known mTOR inhibitor rapamycin inhibits interstitial inflammation, fibrosis, and loss of renal function associated with CKD in a wide variety of animal models [7–13]. Rapamycin exclusively inhibits mTORC1, but does not inhibit mTORC2 [14]. mTORC1 signalling is activated in myofibroblasts from fibrotic kidneys [15] and rapamycin can ameliorate kidney fibrosis by blocking the mTOR signalling in interstitial myofibroblasts, suggesting a possible role for mTORC1 activation in myofibroblasts

* Corresponding author. Dept. of Pathology, H04.312 UMC Utrecht Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Tel.: +31 88 75 56559; fax: +31 88 75 69593.
E-mail address: R.Goldschmeding@umcutrecht.nl (R. Goldschmeding).
[1] Contributed equally.

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in promoting kidney fibrosis [16]. Within lymphocytes, where mTOR is involved in the second phase of T-cell activation, rapamycin blocks IL-2-driven T-cell cycle progression whilst not influencing T-cell survival [17,18]. When rapamycin binds intracellular to its target, the FKBP12 protein, the signal transduction pathway required for the progression of cytokine-stimulated T-cells from the G1 into the S phase is inhibited, thus suppressing interleukin-driven T-cell proliferation.

Rapamycin has great therapeutic potential, but the use of rapamycin and other mTOR inhibitors is associated with many systemic effects. The systemic effects of rapamycin may account for the 20%–40% dropout rate in clinical phase III trials [18]. In rats rapamycin induces decreased food intake and concomitant weight loss [19]. Although some side effects of rapamycin are easily managed, there is an urgent need for renoprotective approaches that are better tolerated. Targeted treatment for the kidney focuses on nanomedicines and conjugates that accumulate in specific kidney cell types such as podocytes [20] and proximal tubular cells [21]. Folate-conjugated rapamycin reduced the progression of polycystic kidney disease in the bpk-mutant mice which illustrates the feasibility of such a prodrug approach [22,23]. We now aim to deliver rapamycin locally in the kidney by subcapsular injection of polymeric microspheres. Rapamycin is a hydrophobic compound that can be formulated efficiently in polymeric devices, which has been investigated extensively for drug-eluting stent [24]. Various other types of biodegradable implants have been loaded with rapamycin, such as poly(L-lactide-co-trimethylene carbonate) matrices [25], perivascular PLGA wraps [26] and PLGA-PEG based thermosensitive hydrogels [27]. Subcapsular injection of a depot under the renal capsule has recently been investigated by Dankers et al. who evaluated the biocompatibility of self-assembling hydrogels and the local release of bone-morphogenetic protein 7 (BMP7) in healthy rats [28–32].

We developed rapamycin polymeric microspheres with a multiblock copolymer consisting of amorphous DL-lactide/polyethylenglycol (PEG)/DL-lactide blocks and crystalline L-lactide blocks. The amorphous PEG-containing blocks favour swelling and gradual erosion of this type of polymeric systems [33]. For the present study we used a block copolymer with 20% w/w of the DL-lactide-PEG-DL-lactide block and 80% of the crystalline L-lactide block. We aimed at a rapamycin loading content of 15% w/w which is much higher than previously reported rapamycin microparticles prepared by emulsification methods which typically contain less than 2% rapamycin [34–36], although a high rapamycin loading content was reported for spray-dried rapamycin-PLGA microparticles for pulmonary delivery [37]. In order to inflict only minimal damage during the injection under the renal capsule, we need to inject the microspheres via narrow needles. Since monodisperse microspheres have a much better syringibility at high concentration we processed the rapamycin polymeric microspheres by microsieving technology [38].

In the present study we explored the feasibility of subcapsular injected microspheres as a drug-eluting depot in the unilateral ureter obstruction (UUO) model in rats. The UUO model is a well-established model for renal fibrosis, in which tubular dilation induces inflammatory and fibrotic cascades discussed above. Local treatment of renal fibrosis with such a depot has not been investigated before, and it is unknown whether drug released from the depot is efficiently distributed throughout the kidney or only active in close proximity to the injection site. We have evaluated the antifibrotic effects of rapamycin by qPCR and immunostaining of fibrosis and mTOR related markers, and have compared the local and systemic effects of subcapsular delivered rapamycin with daily i.p. injections of rapamycin. We hypothesise that local delivery of rapamycin leads to local therapeutic effects with little systemic consequences.

2. Materials and methods

2.1. Formulation of rapamycin microspheres

Polymeric microspheres were prepared using a SynBiosys multiblock copolymer consisting of 20% w/w of poly(DL-lactide-PEG1000) with a molecular weight of 2000 g/mole and 80% w/w poly (L-lactide) with a molecular weight of 4000 g/mole (InnoCore Pharmaceuticals, The Netherlands) (Fig. 1A). Microspheres were prepared by a single emulsion membrane emulsification technique using an Iris-20 microsieve membrane (Nanomi BV, The Netherlands), which is a microfabricated membrane with uniform pores. Placebo (drug-free) microspheres were prepared from a 20% w/v polymer solution in dichloromethane.

Prior to emulsification, the SynBiosys solution was filtered through a 0.2 mm PTFE filter. The membrane-emulsified microparticles were collected into an aqueous solution containing 4% polyvinylalcohol (PVA) as emulsifier. The collected dispersion of microparticles was left to stir at room temperature for at least 3 h to evaporate the solvent. The hardened microspheres were concentrated by filtration and washed repeatedly with ultrapure water containing 0.05% Tween20. For rapamycin-loaded SynBiosys microspheres, rapamycin was co-dissolved with the SynBiosys polymer to achieve a 20% w/w which was used to prepare microspheres as described above. Placebo microspheres and rapamycin-loaded microspheres were stored at −20 °C until evaluation.

2.2. In vitro rapamycin release

Release of rapamycine was studied in triplicate under sink conditions, using 10 mg of rapamycin-loaded microspheres. Samples were suspended in 2.0 ml PBS supplemented with 0.5% SDS and incubated at 37 °C under mild shaking using a shaking water bath. After 0, 1, 3 and 5 h and 1, 2, 5, 8 and 12 days, samples were centrifuged for 1 min at 5000 rpm. Subsequently, 1.8 ml of the supernatant was sampled and refreshed with 1.8 ml of fresh buffer. The amount of released rapamycin was determined by HPLC.

2.3. Unilateral ureteral obstruction

All experiments were performed with the approval of the Experimental Animal Ethics Committee of the University of Utrecht. Female F344/DuCrl rats, weighing 169–196 g, were anesthetized by inhalation of isoflurane (4% induction, 1.5–3% maintenance) and underwent unilateral ureteral obstruction (UUO) of the left kidney by permanent ligation of the ureter. Rats were given carprofen analgesia (0.05 mg/kg subcutaneously) during the first 24 h after surgery with 12-h intervals. Rats were housed in standard cages in a room with constant temperature on a 12 h light-dark cycle. Animals were fed standard animal chow ad libitum and had free access to water.

2.4. Rapamycin therapy

For the analysis of systemic rapamycin therapy, UUO rats were divided in two groups. The first group ($n = 6$) was injected daily intraperitoneally with a vehicle solution (4.8% PEG400, 4.8% TWEEN80, 4.0% ethanol), starting on the day of UUO induction. The second group ($n = 6$) was injected daily intraperitoneally with 2 mg/kg rapamycin in vehicle solution.

For subcapsular microsphere injection we created two triangular subcapsular pockets of 25 μL on the ventral side on the left kidney with a 26G blunt Hamilton needle (Chrom8 International, the Netherlands), that was also used to inject either placebo microspheres (10 mg/50 μL; $n = 6$ rats) or rapamycin-loaded microspheres (10 mg microspheres containing 2 mg rapamycin/50 μL depot; $n = 9$ rats). The microspheres were dissolved in a sterilized carrier solution consisting of 0.6% carboxymethylcellulose, 5% mannitol and 0.1% tween20. After subcapsular injection of the microspheres, the puncture holes in the renal capsule were sealed with fibrin glue (Tissucol©, Baxter, Utrecht, The Netherlands). Rats were sacrificed 7 days after UUO.

2.5. Histology and immunohistochemistry (IHC)

Kidneys were formalin fixed, paraffin-embedded and cut into 3 μm sections. Staining for α-SMA (Sigma, A2457) was performed to assess the accumulation of myofibroblasts. Staining for p-S6 ribosomal protein (Cell Signalling Technology, #2211), a downstream target of mTOR, was used as readout for mTOR activity. Staining for CD3 positive T-Lymphocytes (Dako, A452) was performed to assess the infiltration of CD3 positive T-lymphocytes. Stained sections were scanned (Nikon, Aperio scanscope XT 120) and five 20x random sections per kidney were analysed. In the locally treated groups we scored ten random fields; five at the ventral side of the kidney near the microsphere depot and five at the dorsal side distant from the depot (Fig. 3A). Surface area of α-SMA positive cells and CD3 positive cells was determined using ImageJ (ImageJ, Rasband, National Institutes of Health, USA). p-S6 was scored blind on intensity of staining (arbitrary scale 0–4).