Lymphangiogenesis in renal diseases

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Targeted inhibition of renal Rho kinase reduces macrophage infiltration and lymphangiogenesis in acute renal allograft rejection

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Abstract
The Rho kinase pathway plays an important role in epithelial dedifferentiation and inflammatory cell infiltration. Recent studies suggest that inflammation promotes lymphangiogenesis, which has been associated with renal allograft rejection. We investigated whether targeted inhibition of the Rho kinase pathway in proximal tubular cells reduces inflammation and lymphangiogenesis in acute renal allograft rejection. The Rho kinase inhibitor Y27632 was coupled to lysozyme (Y27632-lysozyme), providing a kidney-specific conjugate that can release its drug in proximal tubular cells. Isogenic (Fisher-Fisher, n=18), or allogenic (Fisher-Lewis, n=24) kidney transplantations were performed, with the contralateral kidney remaining in situ. To elicit acute rejection, no immunosuppressive treatment was given. Animals were treated daily with Y27632-lysozyme (10 mg/kg/day i.v.) or vehicle (saline i.v.) until sacrifice (1 or 4 days post-transplantation).

After allogenic transplantation, interstitial macrophage accumulation was strongly reduced by Y27632-lysozyme at day 4 after transplantation. Interstitial lymphangiogenesis, which was induced in allografts as compared to control kidney, was also reduced by renal Rho kinase inhibition at day 4 after transplantation. The increase of vimentin and procollagen-1alpha1 gene expression in renal allografts from day 1 to day 4 after transplantation was significantly reduced by Y27632-lysozyme. Y27632-lysozyme did not affect systolic blood pressure in isogenic or allogenic transplantation groups. In cultured tubular epithelial cells (NRK-52E), Rho kinase inhibition dose-dependently reduced IL-1β-induced MCP-1 gene expression.

Renal inhibition of Rho kinase causes a marked reduction in renal inflammation and renal lymphangiogenesis during acute transplant rejection, suggesting that this treatment regimen is a valuable future treatment in renal transplantation.
**Introduction**

During the last decades, kidney transplantation has been recognized as the best therapeutic strategy for patients with end-stage renal disease (1). In spite of the progress in surgical techniques, the development of improved immunosuppressive agents, and a better understanding of immunologic phenomena, acute rejection remains a serious complication of kidney transplantation (2). Both cellular and humoral immune responses and many different types of immune cells and cytokines are involved in acute rejection, although the underlying pathophysiologic mechanisms have not been fully elucidated. Activation of Rho-GTPases plays an important role in the regulation of actin cytoskeleton reorganization and inflammation (3, 4). Rho-associated coiled-coil protein kinase (ROCK) is one of the downstream effectors of Rho and has been shown to play a role in inflammation and profibrotic processes in several models of renal damage (5, 6). We therefore postulated that inhibition of the Rho/ROCK pathway might also be beneficial in acute allograft rejection, by reducing renal inflammation. Furthermore, recent studies have identified inflammation-associated de novo renal lymphangiogenesis in human renal transplants (7), and suggested that this process may be associated with renal allograft rejection (8). As acute allograft rejection is characterized by tubular damage (9), it may be advantageous to specifically deliver the kinase inhibitor to renal tubular cells. Besides, cellular targeted delivery of Y27632 to the tubular cells can enhance the efficacy of the drug in the target cells and prevent side effects due to distribution to non-targeted organs (5).

In the present study we investigated whether cell-specific inhibition of the Rho/ROCK pathway in proximal tubular epithelial cells reduces renal inflammation and lymphangiogenesis in a rat model of acute renal allograft rejection.

**Materials and Methods**

**Drug preparation**

To achieve local accumulation of Y27632 in tubular cells of the transplanted kidney, the drug was conjugated to lysozyme, a low molecular weight protein that is filtered in the kidneys and efficiently accumulated in proximal tubular cells. Drug-lysozyme conjugates have been used successfully for the intracellular delivery of small molecule drugs to proximal tubular cells (10). Y27632-lysozyme was prepared as described previously (5). The Rho kinase inhibitor Y27632 [(+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexanecarboxamide dihydrochloride monohydrate] was purchased from Tocris (Bristol, UK). Lysozyme was obtained from Sigma-Aldrich (St. Louis, MO). Cis-[Pt(ethylenediamine)nitrate-chloride] (universal linkage system, ULS™) was freshly prepared from cis-[Pt(ethylenediamine)dichloride as described previously (11). Y27632 (2.95 µmol, 10 mg/ml in water) was basified with 1 M NaOH to pH 8 and reacted with ULS (5.90 µmol) at 50°C overnight. Consumption of the starting drug and formation of the products were followed by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry. Following the reaction, purification was achieved by preparative HPLC. The appropriate fractions were combined, lyophilised, desalted through Sep-Pak column and subsequently eluted with dimethylformamide. Y27632-ULS was subsequently reacted with
Boc-l-methionyl modified lysozyme (0.7 µmol) in 0.02 M tricine/sodium nitrate buffer (pH 8.5). After addition of Y27632-ULS (3.5 µmol), the mixture was reacted at 37°C for 24 h. The product was purified by dialysis against water for 48 h, filtered, lyophilized, and stored at –20°C. Mass spectrometry analysis confirmed the formation of Y27632-lysozyme conjugate. The amount of conjugated drug was quantified after competitive displacement of the drug from the conjugate by overnight incubation with 0.5 M potassium thiocyanate in PBS at 80°C, by HPLC analysis as described previously (5). The absence of free drug in the preparation was investigated by HPLC analysis of freshly prepared dilutions of the conjugate in PBS. Previous studies demonstrated the pharmacokinetics of Y27632-lysozyme and confirmed its selective uptake in proximal tubular cells. Cellular internalization of Y27632-lysozyme occurs via megalin receptor-mediated endocytosis (5).

Animal experimental techniques
Inbred male rats, weighing 215±9 g were used (Harlan, Horst, The Netherlands). Lewis rats (Lew.Ssn) served as recipients and Fisher 344 (F344.NHsd) as donors. F344 kidneys placed in F344 recipients served as controls. Rats were anaesthetized with isoflurane, left donor kidneys were flushed with saline, preserved in saline on ice for 20 minutes and transplanted orthotopically. The left vessels and ureter of the recipient were anastomosed end-to-end using 10-0 Prolene sutures (Johnson & Johnson Intl., Brussels, Belgium). Vascular clamps were released after the vascular anastomosis was completed, with a warm ischemia time of 15 to 18 minutes. The right native kidney was left in situ. Ten percent of the transplantations performed were considered a technical failure due to hydronephrosis and these rats were excluded from the study. To achieve acute rejection of the transplanted kidneys, rats were not treated with cyclosporine or other immunosuppressive drugs. Kidneys from healthy Fisher F344 rats (n=4) were used as controls. All experimental protocols for animal studies followed the national guide for the care and use of laboratory animals, and were approved by the local Animal Ethic Committee of the University of Groningen.

From one day before transplantation until sacrifice, rats received daily intravenous injections with either Y27632-lysozyme (10 mg/kg equivalent to 278 µg/kg of free Y27632) or vehicle (saline i.v.). Animals were sacrificed at day 1 (n=12 allogenic, n=9 isogenic) or at day 4 (n=12 allogenic, n=9 isogenic) after transplantation. Blood pressure was measured at day 0, 1 and 4 after transplantation. A multi-channel computerized system was used with tail cuffs and photoelectric sensors to detect the tail pulse (CODATM; Kent Scientific Corporation, Torrington, CT, USA). Rats were placed in restrainers while the temperature of the tail was maintained at 35 to 37°C. For each rat, the systolic blood pressure value was calculated from the mean of three to five consecutive measurements. Animals were trained for 2 weeks to get adapted to the equipment and procedure.

Determination of mRNA expression
Total RNA was extracted using the TRIZOL method (Invitrogen, Carlsbad, CA). DNase treatment was performed using a Turbo DNA-free kit (Applied Biosystems, Foster City, CA). cDNA was synthesized using Superscript II RT and random hexamer primers (Invitrogen). Relative quantification was performed on an ABI7900HT Taqman (Applied Biosystems). The primers we used were Gene Expression Assays (Applied Biosystems) and hypoxanthine
phosphoribosyltransferase (HPRT) was used as housekeeping gene. The primer reference numbers were as follows: chemokine (C-C motif) receptor 2 (CCR2): Rn 01637698_sl, procollagen1α1: Rn01463848_ml, vimentin: Rn00579738_ml, transforming growth factor-β (TGF-β): Rn00572010_ml, monocyte chemotactic protein-1 (MCP-1): Rn00580555_ml. The following primer sequences were used for HPRT: forward, 5’-GCC.CTT.GAC.TAT.AAT.GAG.CAC.TTC.AA-3’, reverse, 5’-TCT.TTT.AGG.CTT.TGT.ACT.TGG.CTT.TT-3’. SDS2.1 software (Applied Biosystems) was used for data report and calculation. Mean Ct values of the target genes were normalized to the HPRT value (ΔCt) and relative results were expressed as $2^{-ΔCt}$.

**Double-immunofluorescence**

For confirming the identification of lymphatic vessels, double immunofluorescence on acetone-fixed cryostat sections was performed with primary antibodies specific for podoplanin and vascular endothelial growth factor receptor-3 or with lymphatic vessel endothelial hyaluronan receptor-1. Briefly, 4 µm frozen sections fixed in acetone were blocked for endogenous peroxidase activity with 0.03% H2O2 if appropriate. Sections were incubated with primary antibodies against podoplanin (AngioBio, Del Mar, CA), rabbit anti-lymphatic vessel endothelial hyaluronan receptor-1 (Millipore, Billerica, MA) or goat anti-vascular endothelial growth factor receptor-3 (R&D systems, Minneapolis, MN), respectively. Binding of primary antibodies was detected by incubation with secondary antibodies labelled with horseradish peroxidase (DAKO, Glostrup, Denmark) or goat anti-mouse FITC (SouthernBiotech, USA), where appropriate. Horseradish peroxidase activity was visualized using the TSATM Tetramethylrhodamine system (PerkinElmer, Waltham, MA). Nuclei were stained with DAPI. Individual channels were auto-contrasted and overlaid using Photoshop CS5 (Adobe, San Jose, CA).

**Immunohistochemistry**

For the immunohistochemical detection of macrophages (ED1+), lymph vessels (podoplanin+) and vimentin in tissues, 4-µm-thick paraffin-embedded sections were used. Sections were deparaffinized in xylene and rehydrated in alcohol and distilled water. Antigen retrieval was achieved by overnight incubation at 60°C in 0.1 M Tris/HCl buffer (pH 9.0) for ED-1 staining. For vimentin staining, sections were incubated in a microwave for 15 min at 300W with 1mM EDTA (pH 8.0). Endogenous peroxidase activity was blocked with 0.03% H2O2 for 30 min. Slides were then incubated with primary antibodies against ED-1 (Serotec, Oxford, UK), podoplanin (AngioBio) or vimentin (Clone V9, monoclonal mouse anti-vimentin, DAKO). Binding was detected by sequential incubation with peroxidase-labelled secondary and tertiary antibodies in the presence of 1% normal rat serum for 30 min. The peroxidase activity was visualized using 3,3’-diaminobenzidine tetrahydrochloride (DAKO) for 10 min. Sections stained for podoplanin were counterstained with period acid-Schiff and subsequently analyzed for quantification, whereas sections stained for ED1 were quantified first and then counterstained with period acid-Schiff.

**Quantification**

The extent of tubulointerstitial vimentin expression was measured using computerized
morphometry in 30 fields per section at 200x magnification. Glomeruli and vascular areas were manually excluded. Interstitial macrophages were counted in 30 fields per section with a grid at 200x magnification and glomerular macrophages were counted in 50 glomeruli per sections at 400x magnification.

The lymphatic vessel density was quantified as number of podoplanin positive vascular profiles per medium-power field (20X objective). The sections were scanned using a NanoZoomer HT (Hamamatsu Photonics K.K., Shizuoka Pref., Japan). A total of 30 fields per kidney cortex were evaluated and the number of lymph vessel were counted using ImageJ 1.41 (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) which was downloaded from http://rsb.info.nih.gov/ij/download.html.

Cell culture studies
In vitro experiments were performed in a rat tubular epithelial cell line (NRK-52E, American Type Culture Collection (ATCC), Manassas, VA, USA). Cells were grown in DMEM (Gibco, Life technologies, UK) containing 4.5 g/l glucose, supplemented with penicillin (50U/ml) / streptomycin (50 µg/ml), and 5% fetal calf serum, in humidified air at 37 °C with 5% CO2. Cells were seeded in 12-well plates until ±80% confluence; prior to each experiment cells were washed twice with HBSS and starved in serum-free medium for 24h. Subsequently, cells were stimulated with recombinant rat IL-1β (10 ng/ml, R&D Systems, Abingdon, United Kingdom) under serum-free conditions. One hour before stimulation, cells were either pre-incubated with the ROCK inhibitor Y27632 or vehicle. After 6 hrs cells were lysed, mRNA was isolated and realtime qPCR for MCP-1 was performed as described above. Experiments were performed three times.

Statistical analysis
Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA). Differences between groups were calculated using Kruskal Wallis, with P<0.05 as the minimal level of significance. The data are expressed as mean ± standard error of the mean.

Results
Clinical parameters
At time of transplantation, systolic blood pressure was not significantly different between animals treated with the Rho kinase inhibitor Y27632-lysozyme or with vehicle (not shown). Treatment with Y27632-lysozyme did not affect systolic blood pressure in any of the groups, neither at day 1 nor at day 4 after transplantation. The clinical parameters measured in isograft and allograft recipients are summarized in Table 1. Body weight at sacrifice was not different between any of the groups. Serum creatinine levels were not increased in any of the allogenic transplanted groups as compared to isografts, nor did Y27632-lysozyme change serum creatinine as compared to vehicle. Oppositely, in vehicle-treated isografts at 1 day post-transplantation, serum creatinine was slightly higher than other groups (P<0.05 vs vehicle-treated allografts day 1 post-transplantation). At 4 days after transplantation, serum creatinine values were similar among all groups.
Targeted inhibition of renal Rho kinase in acute renal allograft rejection

Figure 1. Effect of Y27632-lysozyme on interstitial and glomerular macrophage accumulation in renal allografts.
(A) Interstitial ED1+ macrophage accumulation was increased in isografts and allografts at day 4 post-transplantation, as compared to healthy control kidneys. Y27632-lysozyme treatment significantly reduced tubulointerstitial macrophage accumulation in allografts 4 days post-Tx. (B) Y27632-lysozyme treatment significantly attenuated glomerular macrophage accumulation in allograft rats at day 4 post-Tx. Y27632-lysozyme did not affect glomerular macrophage accumulation in isografts.

Renal accumulation of macrophages
Allogenic kidney transplantation resulted in an increased accumulation of macrophages in both the tubulo-interstitial (Fig. 1A) and glomerular (Fig. 1B) compartment. At 1 day post-transplantation, tubulo-interstitial macrophage influx was equally low in both isografts and allografts. However, at 4 days post-transplantation tubulo-interstitial macrophage influx was significantly increased in allografts (vs. healthy control kidneys, P<0.05). Treatment with Y27632-lysozyme significantly reduced tubulointerstitial macrophage influx in

Figure 2. Y27632-lysozyme significantly reduced tubulointerstitial ED-1+ macrophage influx 4 days after transplantation.
Representative photomicrographs of renal sections from allografts from vehicle (A: day 1, B: day 4) and Y27632-lysozyme (C: day 1, D: day 4) treated recipients. These images indicate strongly reduced numbers of ED1+ macrophages (dark brown cells) in Y27632-lysozyme -treated recipients 4 days after transplantation (panel D) as compared to vehicle-treated allograft recipients (panel B). Inset: high power magnification of the framed areas shown in panels B & D. Isografts are not shown. Sections were counterstained with PAS. Magnification 200x.
allografts 4 days post-transplantation. Representative photomicrographs of the tubulointerstitium of allografts with or without Y27632-lysozyme treatment are shown in Fig. 2. Glomerular macrophage influx was already increased in allografts 1 day post-transplantation (vs. healthy control kidneys, P<0.05). Treatment with Y27632-lysozyme significantly reduced glomerular macrophage influx in allografts to the level of isografts both 1 and 4 days post-transplantation.

Renal lymphangiogenesis
Specificity of podoplanin as a lymph vessel marker was first confirmed by double-immunolabeling, as demonstrated in Fig. 3. Double-labeling with podoplanin and vascular endothelial growth factor receptor-3 (Fig. 3A) as well as double-labeling with podoplanin and lymphatic vessel endothelial hyaluronan receptor-1 (Fig. 3B) confirmed that podoplanin is a lymph vessel marker. Analysis of the lymph vessels numbers per animal across all groups (Fig. 4) revealed that the number of lymph vessel was increased in allografts at day 1 and day 4 post-transplantation as compared to kidneys from healthy animals. Treatment with Y27632-lysozyme reduced the number of lymph vessels at both time points. Representative photomicrographs of lymph vessel staining are presented in Fig. 5.
Targeted inhibition of renal Rho kinase in acute renal allograft rejection

Renal expression of markers for EMT, inflammation and fibrosis
As demonstrated in Fig. 6A, renal gene expression of vimentin, a marker of epithelial to mesenchymal transformation (EMT), increased in vehicle-treated rats from day 1 to day 4 in both allografts and isografts (P<0.05). In rats treated with Y27632-lysozyme on the other hand, the increase from day 1 to 4 was far less pronounced (P=NS, day 1 vs day 4) both in allografts and isografts. Similarly, renal vimentin protein expression was reduced in rats treated with Y27632-lysozyme as compared to vehicle at day 4 after transplantation (Fig. 6B), although the difference did not reach statistical significance.

Expression of the pro-inflammatory gene MCP-1 increased from day 1 to day 4 in allografts; treatment with Y27632-lysozyme did not affect renal MCP-1 gene expression (Fig. 6C).

Figure 4. Renal lymph vessel numbers are increased in acute rejection at both day 1 and day 4 after allogenic kidney transplantation. Both at day 1 and day 4, treatment with Y27632-lysozyme reduced renal lymphangiogenesis as compared to vehicle-treated allografts at the same time point.

Renal expression of the proinflammatory gene CCR2 was significantly increased in allografts at day 4 after transplantation (relative gene expression 2.10±0.33) as compared to day 1 (0.20±0.03, P<0.05). Treatment with Y27632-lysozyme reduced CCR2 at 4 days after transplantation in allografts (1.74±0.37), but this difference did not reach statistical significance. The fibrotic marker procollagen-1α1 was increased at 4 days after transplantation as compared to day 1 in both allografts and isografts from animals treated with vehicle (Fig. 6D). In Y27632-treated animals, this increase was less pronounced (P=NS vs day 1). Renal TGF-β gene expression also increased in allografts from day 1 (2.05±0.17) to day 4 (4.01±0.26), but Y27632-lysozyme did not affect renal TGF-β gene expression at day 4 after transplantation (3.91±0.14).

Rho kinase inhibition reduces MCP-1 gene expression in cultured tubular epithelial cells
The role of Rho kinase in renal inflammation was further evaluated in cultured rat renal tubular epithelial cells (NRK-52E). After stimulation with IL-1β (10 ng/ml) for 6h, MCP-1 gene expression was strongly (>600 times compared to medium control) increased, while pre-incubation with the ROCK inhibitor, Y27632, dose-dependently reduced IL-1β-induced MCP-1 expression (Fig. 7).
Figure 5. Representative photographs of podoplanin immunohistochemistry, identifying lymph vessels (arrows). (A) Perivascular lymph vessel in a healthy control kidney. (B) Peritubular localization of lymph vessels in healthy control kidneys was very rarely observed. In vehicle-treated allografts, numbers of lymph vessels were increased at 1 day post-transplantation (C), but much more pronounced at day 4 post-transplantation (E). Inhibition of renal Rho kinase by Y27632-lysozyme reduced lymph vessel numbers at both day 1 (D) and day 4 (F) post-transplantation.

Figure 6. Y27632-lysozyme reduced tubular vimentin expression and renal procollagen-1α1 expression 4 days after transplantation.
(A) In both allografts and isografts, renal vimentin gene expression increased from day 1 to day 4 in vehicle-treated rats. In rats treated with Y27632-lysozyme, there was no significant increase in renal vimentin gene expression from day 1 to day 4, neither in allografts nor in isografts. At day 4 after transplantation, vimentin gene expression was reduced in Y27632-lysozyme-treated animals as compared to vehicle-treated animals (both in allografts and in isografts), but this difference did not reach statistical significance. (B) Similarly, renal vimentin protein expression showed a trend to reduction by Y27632-lysozyme. (C) Renal MCP-1 gene expression was not affected by Y27632-lysozyme. (D) In both allografts and isografts, renal procollagen-1α1 gene expression increased from day 1 to day 4 in vehicle-treated rats. In rats treated with Y27632-lysozyme, there was no significant increase in renal procollagen-1α1 gene expression from day 1 to day 4, neither in allografts nor in isografts. At day 4 after transplantation, procollagen-1α1 gene expression was reduced in Y27632-lysozyme-treated animals as compared to vehicle-treated animals (both in allografts and in isografts), but this difference did not reach statistical significance. * P<0.05 vs day 1 (same treatment)
Figure 7. Dose-response curve for the effect of Y27632 on MCP-1 gene expression in cultured tubular epithelial cells (NRK-52E).

Cells were pre-incubated with different doses of Y27632 (black bars) or vehicle (DMSO, white bar) for 1 h and then stimulated with IL-1β (10ng/ml) for 6 hrs. Expression of MCP-1 gene expression was determined by qPCR. The Rho kinase inhibitor Y27632 dose-dependently reduced IL-1β-induced MCP-1 gene expression (P for trend<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>SBP (mmHg) 4 days</th>
<th>SBP, sacrifice (mmHg)</th>
<th>Body weight, sacrifice (g)</th>
<th>Kidney weight, Tx kidney (g)</th>
<th>Kidney weight, contralateral kidney (g)</th>
<th>Serum creat, sacrifice (µmol/l)</th>
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<tr>
<td>Allografts+vehicle 1 day</td>
<td>140±8</td>
<td>125±3</td>
<td>244±21</td>
<td>1.26±0.23</td>
<td>1.23±0.13</td>
<td>32.8±5.0</td>
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<tr>
<td>4 days</td>
<td>142±10</td>
<td>134±7</td>
<td>243±22</td>
<td>1.60±0.38</td>
<td>1.33±0.18</td>
<td>29.7±3.0</td>
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<tr>
<td>Allografts+Y27632-lysozyme 1 day</td>
<td>141±4</td>
<td>154±11</td>
<td>239±24</td>
<td>1.26±0.20</td>
<td>1.21±0.09</td>
<td>33.0±5.7</td>
</tr>
<tr>
<td>4 days</td>
<td>144±5</td>
<td>161±11</td>
<td>228±14</td>
<td>1.63±0.24&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.95±0.13</td>
<td>29.0±2.7</td>
</tr>
<tr>
<td>Isografts+vehicle 1 day</td>
<td>142±4</td>
<td>141±4</td>
<td>228±14</td>
<td>1.11±0.11</td>
<td>1.03±0.16</td>
<td>42.6±8.1</td>
</tr>
<tr>
<td>4 days</td>
<td>144±6</td>
<td>159±10</td>
<td>215±10</td>
<td>1.36±0.37</td>
<td>0.99±0.08</td>
<td>32.0±5.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isografts+Y27632-lysozyme 1 day</td>
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<td>141±4</td>
<td>228±14</td>
<td>1.19±0.20</td>
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</tr>
<tr>
<td>4 days</td>
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<td>161±11</td>
<td>228±13</td>
<td>1.26±0.27</td>
<td></td>
<td>30.4±2.4</td>
</tr>
</tbody>
</table>

Table 1. Clinical parameters

*P<0.05 vs isografts at similar time point (day 1 or day 4) and treatment (Y27632-lysozyme or vehicle), †P<0.05 vs day 1. SBP = systolic blood pressure, Tx kidney= transplanted kidney, Y27632-lysozyme = Rho kinase inhibitor Y27632 coupled to lysozyme

Discussion

Rho kinase activation plays a role in several processes crucial to the pathophysiology of acute renal allograft rejection (i.e. inflammation, ischemia/reperfusion injury). The aim of the current study was therefore to investigate whether renal inhibition of Rho kinase would reduce renal damage in a rat model of acute rejection. Indeed, we found a significantly reduced renal macrophage influx, both in the glomerular and tubulointerstitial compartment. Furthermore, renal Rho kinase inhibition reduced the development of EMT in tubular cells of allogenic transplanted kidneys, and reduced the induction of the fibrotic marker procollagen-1α1 in allografts.

To generate Y27632-lysozyme, the widely used Rho kinase inhibitor Y27632 was chemically coupled to lysozyme, resulting in a conjugate that releases the drug specifically within the proximal tubular epithelial cells of the kidney (5, 12, 13). In follow-up to an earlier study in
which Y27632-lysozyme was evaluated in a renal ischemia-reperfusion injury model, we now investigated its efficacy in kidney allograft transplantation. This study is the first to demonstrate that intrarenal delivery of a Rho kinase inhibitor is an effective strategy to reduce graft-associated inflammation. Together with prior studies in which the systemic efficacy of Y27632 in renal allograft transplantation was shown (14), our study furthermore demonstrates that local activation of tubular Rho kinase plays an important role in macrophage chemoattraction into the renal tubulointerstitium. We also observed an effect of Y27632-lysozyme on glomerular macrophage accumulation, which was slightly but significantly increased compared to healthy control kidneys. This could be explained by tubuloglomerular cross-talk (15, 16); i.e. the reduced tubulointerstitial inflammation by Y27632-lysozyme may subsequently reduce the glomerular influx of macrophages. Similarly to our previous study (5), the absence of effects on blood pressure supports our hypothesis that Y27632-lysozyme acts locally in the kidney without systemic side-effects.

The pathophysiologic role of macrophage accumulation in acute allograft rejection is increasingly recognized (17). Two decades ago, it was demonstrated that in acute allograft rejection, macrophages accumulate in the renal interstitium (18); its abundant presence has been associated with a more severe type of rejection (19). Furthermore, acute vascular rejection is characterized histologically by subendothelial infiltration of mononuclear cells in the arterial intima (20). Besides their immunologic role, macrophages play a fundamental role in tissue remodeling during embryonic development, acquired kidney disease, and renal allograft responses (21). Thereby, the reduction of the procollagen-1α1 gene in response to renal Rho kinase inhibition may be the consequence of reduced renal macrophage accumulation, although the Rho kinase pathway may also directly modulate collagen-1α gene expression (22, 23). Of interest, a recent study demonstrated that leucocytes induce EMT in a model of renal fibrosis (24). Thus, specific Rho kinase inhibition in renal tubular epithelial cells reduced renal macrophage accumulation in acute rejection, which may in turn have reduced EMT and pro-fibrotic (procollagen-1α1) gene expression.

Pro-inflammatory factors are considered to play a central role in macrophage recruitment towards the damaged renal tubulointerstitium (25-27). Although we could not demonstrate a reduction of total kidney MCP-1 mRNA expression by Y27632-lysozyme in our in vivo experiment, we did find a dose-dependent reduction of IL-β-induced MCP-1 expression by the Rho kinase inhibitor (Y27632) in cultured rat tubular epithelial cells. This supports the hypothesis that reduced accumulation of macrophages in our in vivo model was indeed through reduced tubular expression of the chemo-attractant MCP-1.

Beside the effect on macrophage accumulation, Y27632-lysozyme also reduced the number of renal lymph vessels. Our finding that in acute renal allograft rejection the number of lymph vessels is higher than in healthy kidneys is in line with previous studies (8, 28). Of interest, we also found that renal Rho kinase inhibition reduced the numbers of lymph vessels in the kidney. Although the current study does not provide a mechanistic explanation, it could be explained through lower macrophage infiltration in the Y27632-lysozyme-treated group as compared to vehicle. Macrophages may play a major role in lymphangiogenesis, not only by producing high level of chemokines and lymphangiogenic factors (29), but also by incorporating into the lymphatic vessel wall (30). Therefore, reduction of lymph vessel numbers in treated animals compare to controls, could be
secondary to decrease macrophage influx.

**Conclusions**

In conclusion, using a lysozyme-ULS-based drug targeting strategy to deliver the Rho kinase inhibitor Y27632 to renal proximal tubular epithelial cells, we demonstrated reduced macrophage accumulation and lymph vessel density in allografts after induction of acute rejection. Furthermore, the induction of renal vimentin and procollagen-1α1 was reduced by renal Rho kinase inhibition. Rho kinase activation in tubular epithelial cells may play an important role in the tubulointerstitial accumulation of macrophages, and consequently EMT and renal fibrosis, following acute rejection. Renal delivery of Rho kinase inhibitors might be a valuable future treatment in renal transplantation.
References


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