Upregulation of hyaluronan and its binding receptors in an experimental model of chronic cyclosporine nephropathy

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SUMMARY AT A GLANCE
This study provides the first description of the lymphatic vascular endothelial hyaluronan receptor (LYVE-1) in renal fibrosis. The findings raise the possibility that LYVE1 expression by interstitial lymphatic vessels may be involved in the removal of hyaluronan from sites of interstitial fibrosis.

ABSTRACT:
Aim: Hyaluronan (HA) is an important extracellular matrix (ECM) proteoglycan. The localization of HA and its binding receptors, CD44 and LYVE-1, was evaluated in an experimental model of chronic cyclosporine A (CsA)-induced nephropathy.

Methods: Sprague–Dawley rats maintained on a low-salt diet (0.05% sodium) received an s.c. injection of vehicle (1 mL/kg per day olive oil; VH groups) or CsA (15 mg/kg per day; CsA groups) for 1 or 4 weeks. Induction of chronic CsA nephropathy was evaluated according to renal function and pathology and expression of HA, CD44, LYVE-1, ED-1 and α-SMA.

Results: CsA treatment for 4 weeks caused renal dysfunction, which was accompanied by typical striped interstitial fibrosis. In the VH group, HA immunoreactivity was observed only in the inner medulla. However, the area of HA immunoreactivity increased with the duration of CsA treatment: CsA treatment for 1 week extended HA immunoreactivity to the outer medulla, and CsA treatment for 4 weeks caused a further extension of HA immunoreactivity to the cortex, which was vulnerable to CsA-induced renal injury. HA binding receptor, CD44 and LYVE-1 expression were also upregulated in the CsA groups, and were localized to the area of fibrosis and the peritubular capillaries of the cortex. In the CsA groups, ED-1 and α-SMA were predominantly expressed in fibrotic areas in which HA had accumulated.

Conclusion: These findings suggest that upregulation of HA and its binding receptors are involved in interstitial fibrosis in chronic CsA-induced renal injury.

INTRODUCTION
Cyclosporin A (CsA), an effective immunosuppressant, has markedly improved the success of kidney transplantation. However, nephrotoxicity induced by long-term application of CsA causes graft failure, which is a serious problem with organ transplants.1 The most prominent feature of chronic CsA nephrotoxicity is tubulointerstitial fibrosis, which is caused by tubular atrophy, extracellular matrix (ECM) accumulation and thickening of the basement membrane, and results in loss of tubular function.2 However, the mechanism responsible for these effects remains unclear. Recently, several studies have shown that hyaluronan (HA) turnover affects inflammation and fibrosis in injured tissue.3–5

Hyaluronan, a ubiquitous connective-tissue polysaccharide, is an important ECM proteoglycan. It is synthesized predominantly by mesenchymal cells and is present in many tissues.6 Native HA, a high molecular weight polymer, has many biological and pathological effects, including inflammation, angiogenesis, wound healing and tissue remodelling.7,8 To exert its effects, HA must interact with HA-binding proteins. It is well known that CD44 is the major cell-surface HABP.9,10 It is a single-pass transmembrane glycoprotein and is expressed in the ECM and on the surfaces of many cell types, including fibroblasts, neutrophils, macrophages and lymphocytes.11 CD44 has a wide variety of cellular functions, including cell–cell aggregation, retention of pericellular
matrix, receptor-mediated internalization/degradation of hyaluronan, and activation of immune cells and promotion of fibrosis during wound healing.\(^2,15\) Recently, a novel HA binding receptor, lymphatic vascular endothelial HA receptor (LYVE-1), was detected in lymphatic vascular endothelial cells and the tubular cuboidal epithelium of the kidney.\(^15\) LYVE-1 has been identified as a major receptor for HA on the lymph vessel wall. The deduced amino acid sequence of LYVE-1 predicts a 322-residue type I integral membrane polypeptide with 41% similarity to the CD44 HA receptor with a 212-residue extracellular domain containing a single Link module, the prototypic HA binding domain of the Link protein superfamily. Like CD44, the LYVE-1 molecule binds both soluble and immobilized HA. However, unlike CD44, the LYVE-1 molecule co-localizes with HA on the luminal face of the lymph vessel wall and is absent from blood vessels. LYVE-1 plays a role in transporting HA from tissue to lymph by transporting HA into lymphatic endothelial cells.\(^15\)

Based on previous findings, we hypothesized that increased renal tubulointerstitial fibrosis may correlate with expression of HA. Therefore, in this study, we observed the expression of HA and its binding receptors, CD44 and LYVE-1, in chronic CsA nephropathy.

**METHODS**

**Animals and drugs**

The Animal Care Committee of the Catholic University of Korea approved the experimental protocol and all procedures were conducted in accordance with the Ethical Guidelines for Animal Studies. Male Sprague–Dawley rats weighing 225–250 g were housed in individual cages in a temperature- and light-controlled environment at the Catholic University’s animal care facility. The rats received a low-salt diet (0.05% sodium; Teklad Premier, Madison, WI, USA). CsA was provided by Novartis Research (Novartis Pharma, Basel, Switzerland). CsA was diluted with olive oil (Sigma, St Louis, MO, USA) to a final concentration of 15 mg/mL.

**Experimental design**

Rats were randomized into four groups of six rats each and received the treatments for 1 or 4 weeks. The vehicle (VH) groups received daily s.c. injections of olive oil (1 mg/kg) for 1 or 4 weeks and the CsA groups received daily s.c. injections of CsA (15 mg/kg) for 1 or 4 weeks.

**Measurement of basic parameters**

Rats were pair-fed, and bodyweight (BW) was monitored daily after the treatments had been applied. After treatment with CsA for 1 or 4 weeks, animals were anaesthetized with ketamine and blood and kidney samples were obtained. Serum creatinine (Scr), blood urea nitrogen (BUN) and CsA concentrations were measured using an autoanalyzer (Coulter-STKS; Coulter Electronics, Hialeah, FL, USA).

**Preservation of kidneys**

Kidneys were preserved by in vivo perfusion through the abdominal aorta. The animals were perfused with 0.01 mol/L phosphate-buffered saline (PBS) to flush blood from the tissues. A kidney was removed after perfusion with periodate–lysine–paraformaldehyde (PLP) solution. The kidneys were cut into sagittal slices and postfixed overnight in PLP solution at 4°C. Portions of kidney were embedded in wax for histopathological staining and post-embedding immunohistochemistry, and the other portions were used for pre-embedding immunostaining.

**Histopathology**

Sections (5 µm thick) were dewaxed with xylene, hydrated with 100%, 95%, 90%, 80% and 70% ethanol gradually, and Masson trichrome staining was performed to measure tubulointerstitial fibrosis (TIF). TIF was defined as a matrix-rich expansion of the interstitium with tubular dilatation, atrophy and cast formation, and sloughing of tubular epithelial cells or thickening of the tubular basement membrane. At least 20 fields per section were assessed using a colour-image analyzer (TDI Scope Eye ver. 3.0 for Windows; Olympus, Tokyo, Japan). The extent of TIF was quantified using the Polygon program (Image Partnership, Gyeyonggi, Korea) to determine the area of a ×200 field that consisted of injured cortex. Histopathological analysis was performed using randomly selected cortical fields of sections by a pathologist blinded to the identity of the treatment groups.

**Post-embedding immunohistochemistry**

Dewaxed and hydrated tissue sections (5 µm thick) were incubated in 0.5% Triton X 100/PBS solution for 30 min and washed three times with PBS. Non-specific binding sites were blocked using normal horse serum diluted 1:10 in 0.3% bovine serum albumin for 30–60 min and then incubated in a humid environment for 2 h at 4°C in biotin-conjugated hyaluronic acid binding protein (b-HABP) (Seikagaku, Tokyo, Japan). After rinsing in Tris-buffered saline (TBS), sections were incubated in avidin–biotin coupled peroxidase conjugated rabbit antirabbit immunoglobulin (Ig)G (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 30 min. For coloration, sections were incubated with a mixture of 0.05% 3,3′-diaminobenzidine (DAB) containing 0.01% H2O2 at room temperature until a brown colour was visible, washed with TBS, counterstained with haematoxylin, and examined using light microscopy.

Co-localization of HA and ED-1 (anti-CD68, monocytes, and macrophages; Abcam, Cambridge, UK) and HA and α-smooth muscle actin (α-SMA; Abcam, Cambridge, UK) was observed using double immunohistochemistry. HA-stained sections were incubated with primary antibodies (ED-1 or α-SMA) overnight at 4°C, and then the primary antibodies were detected using avidin–biotin coupled peroxidase-conjugated rabbit antirabbit IgG or peroxidase-conjugated goat antirabbit IgG (Amersham Pharmacia Biotech). The tissues were rinsed in washing buffer and then in 0.05 mol/L Tris buffer, pH 7.6. For visualization of horseradish peroxidase, sections were again incubated with the substrates (DAB for HA and vector SG for ED-1 and α-SMA).

To identify CD44 expressing cell types, 5 µm thick sections were double stained with CD44 (Abcam) and CD8 (Santa Cruz Biotech-
BUN (mg/dL) 16.5
CsA (ng/mL) 1761
Scr (mg/dL) 0.51

DAB (5,5′-diaminobenzidine) was used to visualize protein expression.

**Table 1** Basic functional parameters of the treatment groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>VH1</th>
<th>CsA1</th>
<th>VH4</th>
<th>CsA4</th>
</tr>
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<tbody>
<tr>
<td>BW (g)</td>
<td>296 ± 3</td>
<td>298 ± 2</td>
<td>314 ± 2</td>
<td>286 ± 3*†</td>
</tr>
<tr>
<td>Scr (mg/dL)</td>
<td>0.51 ± 0.03</td>
<td>0.62 ± 0.02</td>
<td>0.55 ± 0.02</td>
<td>0.85 ± 0.08*†</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>16.5 ± 0.7</td>
<td>24.4 ± 1.9*</td>
<td>16.2 ± 0.3</td>
<td>55 ± 8.1*†</td>
</tr>
<tr>
<td>CsA (ng/mL)</td>
<td>1761</td>
<td></td>
<td></td>
<td>2812 ± 49†</td>
</tr>
</tbody>
</table>

Values are means ± standard error of the mean (n = 6 for each group). VH1 and VH4, vehicle-treated for 1 or 4 weeks; CsA1 and CsA4, cyclosporine A-treated for 1 or 4 weeks; BUN, blood urea nitrogen; BW, bodyweight; CsA, blood cyclosporine A; Scr, serum creatinine. *P < 0.05 versus VH, †P < 0.05 versus CsA1.

**Pre-embedding immunohistochemistry**

Vibratome sections (50 µm thick) were incubated for 3 h with 1% bovine serum albumin (BSA), 0.05% saponin and 0.2% gelatin-PBS (solution B). The tissue sections were then incubated overnight at 4°C with primary antibodies (CD44, Abcam; LYVE-1, Boster, Wuhan, China) in 1% BSA-PBS (solution A). After several washes with 0.1% BSA, 0.05% saponin and 0.2% gelatin-PBS (solution C), the tissue sections were incubated for 2 h with peroxidase-conjugated secondary antibodies goat antirabbit IgG or donkey anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The tissues were rinsed in solution C and then in 0.05 mol/L Tris buffer, pH 7.6. For visualization of horseradish peroxidase, sections were incubated with substrates (DAB). The tissue sections were dehydrated in a graded series of ethanol, and embedded in poly/Bed 812 resin (Polysciences, Warrington, CA, USA). Sections (1 µm) were cut, and the tissue sections were examined with light microscopy and photographed with an Olympus photomicroscope.

**Immunoblot analysis**

Protein was extracted with lysis buffer (Promega). Protein (20 µg) from each sample were separated on a 10% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% skimmed milk for 1 h and incubated with polyclonal primary antibodies against the CD44 and LYVE-1, overnight at 4°C. After washing with washing buffer (0.3% Tween-20 in PBS), the membranes were incubated with horseradish peroxidase-conjugated donkey antirabbit and donkey antigoat IgG (DAKO, Tokyo, Japan) for 1 h at room temperature. The membranes were developed with an ECL Western blotting system (Amersham Biosciences Korea, Seoul, Korea) and then exposed to X-ray film. Intensity of each band was measured with densitometric scanning analysis (TINA ver. 2.10e; Raytest Isotopenme geräte, Staufenhardt, Germany). Relative density was presented as a percentage of the VH group and normalized with β-actin.

**Statistical analysis**

Data are expressed as means ± standard error of the mean. Multiple comparisons among groups were performed using one-way ANOVA and the post-hoc Bonferroni’s test (SSPS software ver. 9.0; SPSS, Chicago, IL, USA). Statistical significance was accepted at P < 0.05.

**RESULTS**

**Induction of chronic CsA nephropathy**

Table 1 shows values for basic functional parameters. Bodyweight was less in the CsA4 group than in the VH group. Scr level was significantly greater in the CsA4 group than in the VH4 group. BUN level was significantly elevated in the CsA1 and CsA4 groups. Compared with the CsA1 group, the values of all parameters were elevated in the CsA4 group (Table 1). Histological assessment showed a significant increase in tubulointerstitial fibrosis in the CsA1 and CsA4 groups compared with that in the VH1 and VH4 groups. The TIF was much greater in the CsA4 group than in the CsA1 group (Fig. 1).

**Expression of hyaluronan in CsA-induced renal injury**

Figure 2 shows that HA immunoreactivity was greatest in the inner medulla and gradually decreased from the inner medulla to the cortex in normal rat kidney. HA was mainly observed in the interstitium of the outer and inner medulla. In the cortex, HA was observed in a few vessels. However, there was no HA immunoreactivity in the tubules of the kidney. Compared with the VH groups, HA expression in the CsA groups increased in a time-dependent manner. The expression of HA increased in the inner and the outer medullas of the CsA1 group and extended to the whole layer in the CsA4 group. However, the increase in HA expression in the cortex was the most remarkable (Fig. 2). High-resolution images of HA immunoreactivity are shown in Figure 3. The expression of HA did not differ between the VH1 group and CsA1 group in the inner stripe of the outer medulla, but was greater in the outer stripe of the outer
Fig. 1 Cyclosporine A (CsA)-induced nephropathy. Trichrome staining was used for assessing the tubulointerstitial fibrosis (TIF) (A) (original magnification ×200). The bar graph shows the percentage of injured areas per field in the cortex (B). Values are means ± standard error of the mean. *P < 0.05 vs vehicle (VH) groups; †P < 0.05 vs CsA1 group.

Fig. 2 Expression of hyaluronan (HA) in kidneys of cyclosporine A (CsA)-treated rats. Light micrographs of 5 μm thick microtome sections from rat kidney illustrating HA immunoreactivity in the entire kidney for the vehicle (VH1, CsA1, VH4 and CsA4) groups. Cx, cortex; OM, outer medulla; IM, inner medulla. (Original magnification ×40.)
Expression of HA was elevated in the cortex and the whole of the medulla of the CsA4 group.

Expression of CD44 and LYVE-1 in the renal cortex

Figure 4 shows the expression of CD44 and LYVE-1 in the cortex of the kidney. In the VH groups, CD44 was seldom expressed in the peritubular interstitial cells of the cortex. In the CsA1 group, CD44 expression was significantly elevated in the basolateral membrane of damaged tubules of the cortex. However, the expression of CD44 in the CsA4 group was significantly elevated in the fibrotic area of the cortex. Expression of LYVE-1 in the VH group was observed in the capillaries of the peritubular area of the cortex. However, in the CsA1 and CsA4 groups, expression of LYVE-1 was significantly elevated in the capillaries of the peritubular areas of the cortex. The expression of CD44 and LYVE-1 was much greater in the CsA4 group than in the CsA1 group (Fig. 4A).

Identification of cell types expressing LYVE-1 and CD44

Figure 5(A,B) show the localization of LYVE-1 and Poloplin in rat kidney. In two consecutive sections (Fig. 5A,B), it was showed that most LYVE-1 expressing vessels and podoplanin expressing lymphatic vessels were overlapped. Figure 5(C) shows that the CD44 expressing cells were also presented CD8 (Fig. 5C).

Expression of ED-1 and α-SMA in the cortex of the kidney

Figure 6 shows the co-localization of HA and ED-1, and HA and α-SMA. In the CsA-treated groups, there was a marked increase in the infiltration of ED-1-positive macrophages into
Fig. 4 Expression of CD44 and LYVE-1 in the renal cortex of the vehicle (VH) and cyclosporine A (CsA) groups. Light micrographs of 5 μm thick microtome sections from rat kidney illustrating CD44 and LYVE-1 immunoreactivity in the cortex (A). The arrows indicate CD44 expression in the peritubular interstitium of the cortex. The open arrows indicate the LYVE-1 immunoreactivity in the peritubular capillary of the cortex. (Original magnifications: CD44, ×400; LYVE-1, ×1000.) Expression of LYVE-1 and CD44 were detected by immunoblot (B). Relative density of LYVE-1 and CD44 were presented as a percentage of VH group and normalized with β-actin (C,D). The results are expressed as the mean ± standard error of the mean (n = 4). *P < 0.05 vs VH group; #P < 0.05 vs CsA group.
inflamed areas that expressed HA. Levels of α-SMA, a marker of myofibroblasts, were also elevated in the peritubular basement membranes of damaged tubules and fibrotic areas of cortex in the CsA-treated groups. The expression of ED-1 and α-SMA was much greater in the CsA4 group than in the CsA1 group.

DISCUSSION
In this study, animals treated with CsA developed renal dysfunction. Tubulointerstitial fibrosis and expression of HA and its binding receptors, CD44 and LYVE-1, increased simultaneously in the damaged kidneys. The increase in the expression of HA and its binding receptors was time-dependent and was localized to areas of interstitial fibrosis. This suggests that an interaction between HA, CD44 and LYVE-1 facilitated the pathogenesis of chronic CsA-induced nephropathy.

In the normal kidney, HA was mainly present in the inner medulla, but was not detected in renal tubular cells. These results support a previous report showing that most HA synthesis emanates from interstitial cells.16,17 Its preferential
localization in the inner medulla suggests that it plays a role in urinary concentration. However, renal HA immunoreactivity in the CsA groups was high and occurred over a large area. The immunoreactivity of HA in the CsA groups increased in the outer medulla 1 week after treatment was commenced and extended to the cortex after 4 weeks of treatment. HA immunoreactivity was elevated in the interstitium of the outer medulla and the interstitial fibrotic areas of the cortex, which is similar to observations using the ischemia/reperfusion (I/R)-induced inflamed kidney. HA is not a major constituent of the corticointerstitium of normal kidneys. However, both cortical and medullary fibroblasts are capable of synthesizing HA \textit{in vitro}. Numerous studies have demonstrated that increased expression of HA in the renal corticointerstitium is associated with progressive renal diseases. Furthermore, in progressive renal disease, alterations in HA expression are correlated with the degree of interstitial fibrosis and decline in renal function. Increased expression of HA is also associated with acute reversible ischemic renal injury. In contrast, HA limits scar formation and tissue remodelling in wound healing. From these reports, it is suggested that altered HA synthesis and accumulation within the kidney, and leucocyte activation and turnover in the kidney may either be involved in the repair and maintenance of normal homeostasis or in the development of progressive renal scarring. In our study, increased co-localization of HA and \( \alpha-SMA \) in areas of fibrosis indicates that HA is associated with CsA-induced renal fibrosis.

CD44, a HA receptor, is the major cell-surface HABP, and is expressed in immune cells. HA–CD44 interactions may facilitate leucocyte-mediated renal injury, including leucocyte recruitment from the circulation, leucocyte migration and accumulation within the kidney, and leucocyte activation. This concept is supported by a recent \textit{in vitro} study, which showed that when expressed on T cells, CD44 interacts with HA on the surface of the endothelium and facilitates lymphocyte rolling under physiological flow conditions. The pathogenic role of CD44 (and HA) in inflammation and renal fibrosis has been shown in mice models of I/R injury and obstructive nephropathy using knockout mice. Similarly, we observed that CD44 expression was increased in CsA-treated animals and was localized in the peritubular basolateral membranes of damaged kidneys. Furthermore, CD44 is involved in interstitial inflammation. Consistent with this, we observed an increase in macrophage numbers in areas which HA had accumulated in. Based on these findings, we speculate that LYVE-1 is upregulated in the inflammatory vessels are important for wound healing in diabetics. In this study, we found that HA production was markedly increased in inflamed and fibrotic areas. Based on these findings, we speculate that LYVE-1 is upregulated in the capillaries of the peritubular interstitium to transport overexpressed HA to the lymph and may be associated with reparative fibrosis in chronic CsA nephropathy.

In conclusion, we studied the relationship between HA and CsA-induced nephrotoxicity and found that HA and its binding receptors are involved in interstitial inflammation and fibrosis in chronic CsA-induced nephropathy. Further studies should be conducted to identify the mechanisms responsible for the interactions between HA and other factors.

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