Transforming growth factor-β induces vascular endothelial growth factor-C expression leading to lymphangiogenesis in rat unilateral ureteral obstruction

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Inflammation is recognized as an important contributor to lymphangiogenesis; however, in tubulointerstitial lesions in human chronic kidney diseases, this process is better correlated with the presence of myofibroblasts rather than macrophages. As little is known about the interaction between lymphangiogenesis and renal fibrosis, we utilized the rat unilateral ureteral obstruction model to analyze inflammation, fibrosis, lymphangiogenesis, and growth factor expression. Additionally, we determined the relationship between vascular endothelial growth factor-C (VEGF-C), an inducer of lymphangiogenesis, and the profibrotic factor, transforming growth factor-β1 (TGF-β1). The expression of both TGF-β1 and VEGF-C was detected in tubular epithelial and mononuclear cells, and gradually increased, peaking 14 days after ureteral obstruction. The kinetics and localization of VEGF-C were similar to those of TGF-β1, and the expression of these growth factors and lymphangiogenesis were linked with the progression of fibrosis. VEGF-C expression was upregulated by TGF-β1 in cultured proximal tubular epithelial cells, collecting duct cells, and macrophages. Both *in vitro* and *in vivo*, the induction of VEGF-C along with the overall appearance of lymphatics *in vivo* was specifically suppressed by the TGF-β type I receptor inhibitor LY364947. Thus, TGF-β1 induces VEGF-C expression, which leads to lymphangiogenesis.

Kidney International (2012) **81,** 865–879; doi:10.1038/ki.2011.464; published online 18 January 2012

KEYWORDS: LYVE-1; podoplanin; TGF-β; tubular epithelial cells

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Received 11 January 2011; revised 5 October 2011; accepted 1 November 2011; published online 18 January 2012

Blood vessels have a continuous basal lamina with tight inter-endothelial junctions and are supported by pericytes and smooth muscle cells. In contrast, lymphatic endothelial cells have a thin discontinuous basement membrane and have gaps between the cells that open to the adjacent connective tissues.[1] In edematous tissue, the lymphatic endothelial cells are pulled by anchoring filaments and bileaflet valves to prevent the backflow of lymphatic fluid.[2] These structures remove tissue fluid from the interstitium and transfer extravasated plasma protein and cells back into circulation. In this respect, lymphatic vessels are essential for body fluid balance and immunological surveillance.[3]

Increases in lymphatic vessels have recently been reported in several disease conditions, including tumor metastasis,[4–7] chronic respiratory inflammatory diseases,[8] wound healing,[9] renal transplant rejection,[10,11] and granulation tissues in myocardial infarction.[12] Inflammation is recognized as an important contributor to lymphangiogenesis in human diseases[10,13] and in animal models.[14,15] In particular, macrophages are involved in lymphangiogenesis in the production of vascular endothelial growth factor (VEGF)-C and -D, which are recognized as potentially important mediators for lymphangiogenesis.[16] Interleukin-1β and tumor necrosis factor-α have been shown to induce the upregulation of VEGF-C.[17] In addition, CD11b+ macrophages may form lymphatic-like vessels *in vitro*.[14,18] A recent report showed that inflammation induced lymphangiogenesis through the upregulation of VEGF receptor-3 mediated by nuclear factor-kappa-B and prospero-related homeobox 1.[19]

In chronic kidney disease, we recently reported an increase in the number of lymphatics observed at the site of tubulointerstitial lesions and this increase was correlated with

degree of tissue damage. In addition, lymphangiogenesis was more strongly correlated with fibrosis than inflammation on analysis of human glomerular diseases and acute and chronic tubulo-interstitial nephritis.[20] We also demonstrated strong expression of VEGF-C in the proximal tubules.[20] Our results indicated that lymphangiogenesis is a common feature in the progression of tubulo-interstitial fibrosis, and the fibrotic process may play a role in the development of lymphatics.[20] However, there has been little focus on the role of transforming growth factor (TGF)-β, one of the most important mediators for tissue fibrosis, in lymphangiogenesis to date.

In this study, we investigated the roles of TGF-β and VEGF-C in the development of lymphangiogenesis in the unilateral ureteral obstruction (UUO) model. In addition, we studied the relationship between TGF-β and VEGF-C in cultured proximal and collecting tubules, macrophages and fibroblasts, which are involved in tubulo-interstitial fibrosis. This is the first report to examine the mechanisms and roles of TGF-β in lymphangiogenesis.

RESULTS

Basic characteristics of the rat UUO model

Three days after ligation, dilation of the tubules and infiltration of inflammatory cells were seen in both the cortex and the medulla. On day 14, tubular degeneration, dilation, and atrophy became severe and intense interstitial fibrosis was also observed (Figure 1). Morphological analysis demonstrated strong infiltration of CD68 (ED-1)-positive macrophages in both the cortex and medulla on day 3 that was more prominent than the expression of α-smooth muscle actin (α-SMA)-positive fibroblasts (Figure 2a and b). Expression of α-SMA-positive fibroblasts and type III collagen deposition in the tubulo-interstitial area became conspicuous and peaked on day 14 (Figure 2).

Lymphatic vessels in control and UUO model

In the control kidney, podoplanin-positive and lymphatic endothelial hyaluronan receptor-1 (LYVE-1)-positive lymphatic vessels were not encountered in the normal cortical tubulo-interstitial area and were observed only

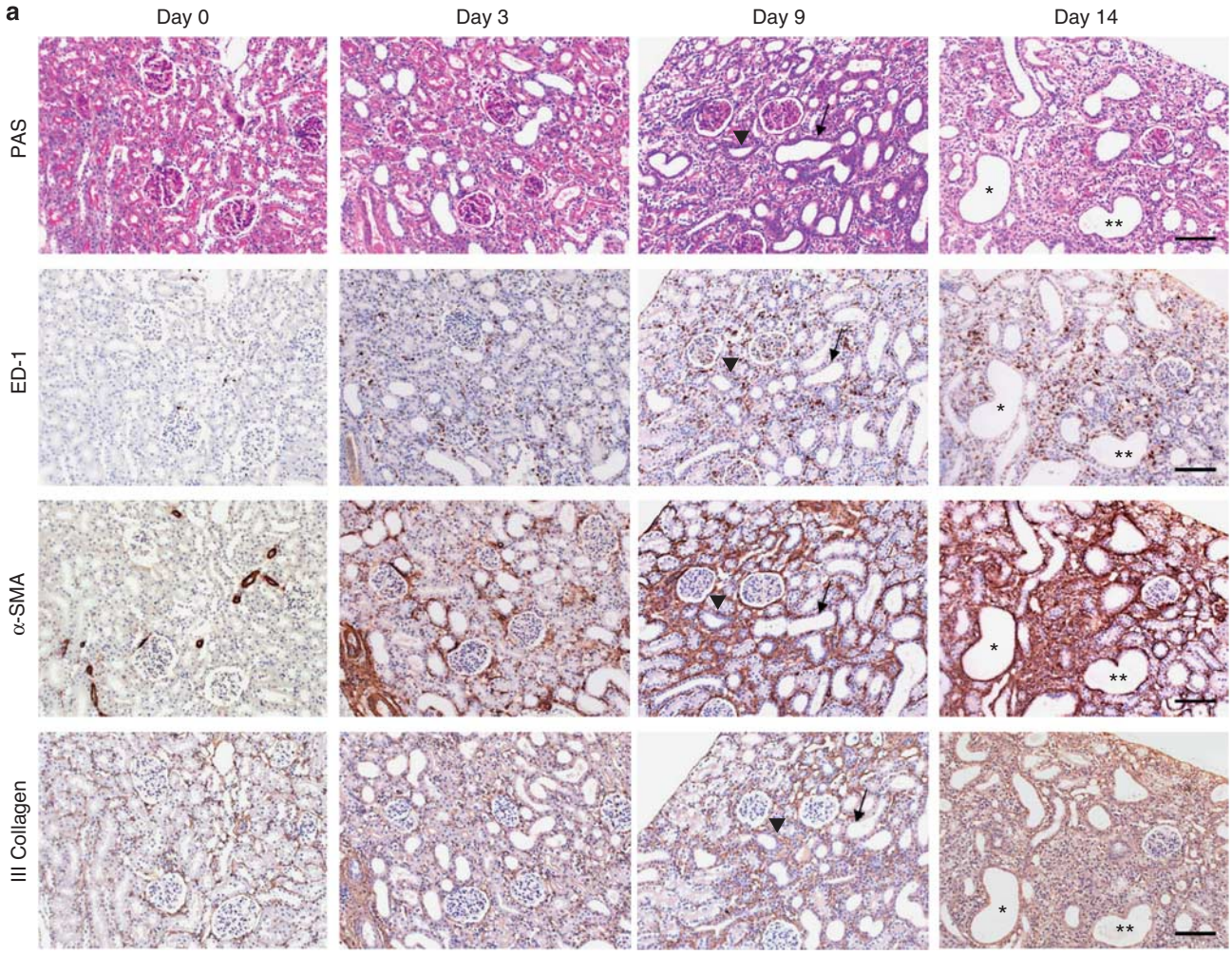

Figure 1 | Rat unilateral ureteral obstruction (UUO) model. (**a**) Renal cortex of rat UUO model. (**b**) Renal medulla of rat UUO model. Arrows, arrowheads, asterisks, triangles, and stars indicate the same dilated tubules. Bar = 100 μm. ED-1, CD68; P, renal pelvis; PAS, periodic acid–Schiff; α-SMA, α-smooth muscle actin.

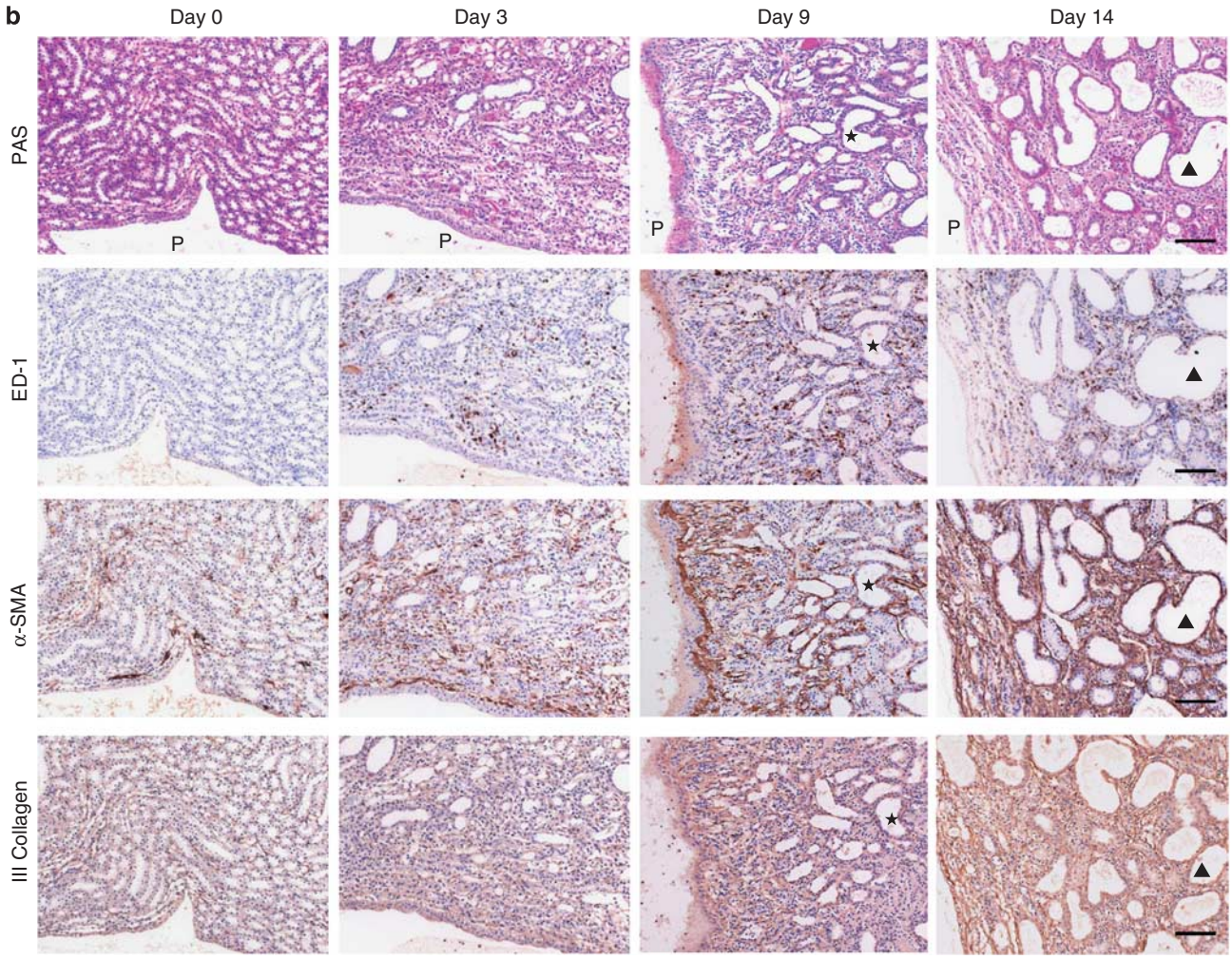

Figure 1 | Continued.

adjacent to the large and intermediate-sized vessels (Figures 3a, b, and 4). In the glomeruli, podoplanin was expressed in the podocytes and parietal epithelial cells. In contrast, LYVE-1 was expressed in the glomerular endothelial cells detected by anti-aminopeptidase P antibody, a marker of rat endothelial cells,[21] on serial sections and double staining (Figure 3a–c, Supplementary Figure S1a–c online). In the kidney with ureteral obstruction, podoplanin- and LYVE-1-positive lymphatic vessels grew with time and peaked on day 14 in both the medullary and cortical areas (Figures 3d–g and 4). On serial sections of the medulla on day 14, a number of podoplanin- and LYVE-1-positive lymphatic vessels that were negative for aminopeptidase P were observed, and the entry of the lymphatics was confirmed in the renal pelvis (red and blue arrows in Figure 3h–j). On the other hand, endothelial cells of the blood vessels were negative for podoplanin and LYVE-1 (Figure 3h–j). The expression pattern of podoplanin in the interstitial area was similar to that of LYVE-1 (Supplementary Figure S1d–i). The expression pattern in the glomeruli of these three markers (Figure 3h–j) was the same as those in control kidneys (Figure 3a–g).

Expression of VEGF-C and TGF-β, and lymphangiogenesis during UUO

Because TGF-β is known to have an important role in the pathogenesis of tubulo-interstitial fibrosis,[22] expression of TGF-β was compared with that of VEGF-C in this model. On immunohistochemistry, expression of TGF-β protein detected by anti-TGF-β 1, 2 and 3 antibodies was seen to increase gradually, peaking on day 14 (Figure 4); this expression was primarily seen in the tubular epithelial cells, and was partly seen in the inflammatory cells. TGF-β expression in the glomeruli was not elevated. In the control kidney, VEGF-C was weakly detected in the tubular epithelial cells (day 0, Figure 4). VEGF-C expression was also gradually upregulated, mainly in the enlarged tubular epithelial cells in both the cortex and medulla after disease induction. Although we were able to detect VEGF-C in the infiltrating cells in the tubulo-interstitial area on days 9 and 14, expression was more marked in the tubules than in the inflammatory cells (Figure 4, Supplementary Figures S2–4 online). Morphometric analysis demonstrated that TGF-β and VEGF-C expression paralleled lymphangiogenesis (Figure 5).

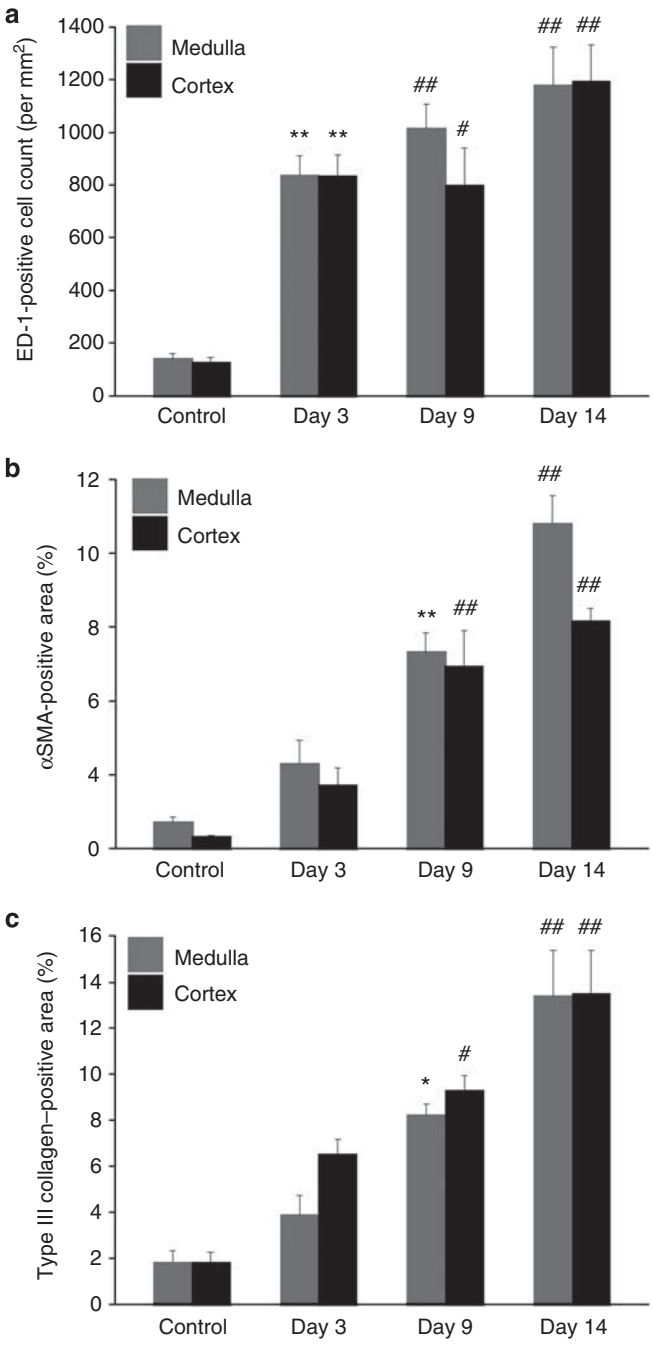

Figure 2 | Quantification of morphological analysis by immunohistochemistry in rat unilateral ureteral obstruction. CD68 (ED-1)-positive macrophage infiltration (**a**) increased significantly on day 3 and continued to increase until day 14. Areas positive for α-smooth muscle actin (α-SMA) (**b**) and type III collagen (**c**) increased with time and peaked on day 14. Values are means ± s.e. ($N = 5$). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{\#}P < 0.01$; $^{\#\#}P < 0.001$ vs. control.

Localization of VEGF-C and TGF-β protein in UUO (day 14)

To identify the segments of tubules that expressed VEGF-C and TGF-β, serial sections were stained with aquaporin-1 and aquaporin-2. VEGF-C and TGF-β were expressed by the aquaporin-1-positive proximal tubules and aquaporin-2-positive collecting tubules in the injured tubulo-interstitial areas of the cortex and the medulla (Figure 6). Interestingly, localization of VEGF-C was similar, and often showed co-localization with TGF-β (Figure 6, Supplementary Figure S3 online).

VEGF-C, TGF-β, podoplanin, and LYVE-1 messenger RNA (mRNA) expression analyzed by real-time polymerase chain reaction (PCR) in UUO

All isoforms of TGF-β and VEGF-C mRNA increased with time, and peaked on day 14 in both the cortex and medulla (Figure 7a–d). mRNA expression of LYVE-1, podoplanin, and vascular endothelial growth factor receptor-3, markers of lymphangiogenesis, increased over time and peaked on day 14 (Figure 7e–h, Supplementary Figure S5a and b online). These kinetics were similar to those of TGF-β and VEGF-C mRNA and protein (Figures 5a, b, and 7a–d). These expression patterns were also comparable to the expression of α-SMA and type III collagen, but not ED-1-positive macrophages (Figure 2). These findings indicate that lymphangiogenesis developed in association with the fibrotic process. In contrast, the expression of VEGF-A mRNA, the most potent mediator of neoangiogenesis, was slightly lower on day 3, but was not significantly altered during the observation period (Supplementary Figure S6 online).

VEGF-C induction by TGF-β1 in cultured proximal tubular epithelial (HK-2) cells, collecting duct (M-1) cells, macrophages (RAW264.7) and fibroblasts (NRK-49F)

To explore the roles of TGF-β in lymphangiogenesis, we investigated VEGF-C induction by TGF-β1 in cultured proximal tubular epithelial cells, collecting duct cells, macrophages and fibroblasts, which are involved in the development of tubulo-interstitial injury of UUO (Figure 8).

In human kidney-2 (HK-2, human proximal tubular epithelial cells), dose–response studies were conducted using incubation for 24 h with recombinant TGF-β1 at 0, 1, 5, or 20 ng/ml. TGF-β1 induced significant upregulation of VEGF-C protein ($P < 0.001$), determined by an enzyme-linked immunosorbent assay and VEGF-C mRNA expression ($P < 0.001$), determined by real-time PCR (Figure 8a and c). On the basis of these experiments, time-course studies were performed using 5 ng/ml TGF-β1. VEGF-C mRNA/18S ribosomal RNA increased by about a 2- to 2.5-fold and peaked at 12 h. VEGF-C protein was secreted into supernatants under serum-free conditions and without TGF-β incubation. TGF-β1 significantly induced VEGF-C protein secretion at 24 h ($P < 0.001$, Figure 8b).

In the M-1 mouse cortical collecting duct cell line, TGF-β1 induced the upregulation of VEGF-C mRNA expression. VEGF-C mRNA/18S ribosomal RNA increased and peaked 24 h after incubation with 5 ng/ml TGF-β1 (Figure 8e and f).

In mouse macrophages (RAW264.7), concentrations above 5 ng/ml TGF-β1 significantly increased VEGF-C mRNA expression ($P < 0.05$, Figure 8g). VEGF-C mRNA/18S ribosomal RNA was elevated and peaked at 12 h after incubation with 5 ng/ml TGF-β1 (Figure 8h). VEGF-C induction by

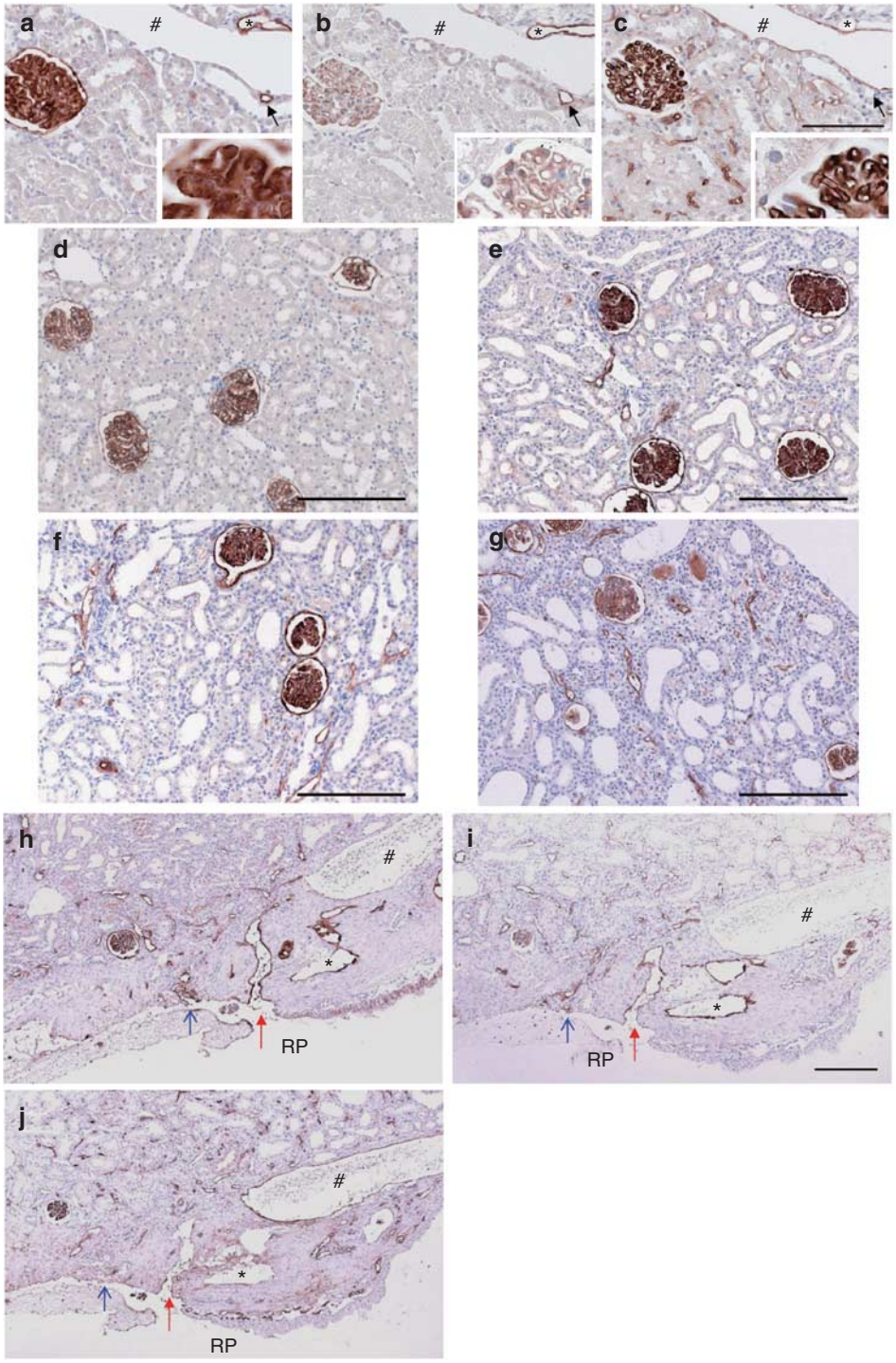

Figure 3 | Lymphatic vessels in normal and obstructed kidneys. (**a**–**c**) Immunohistochemical staining for podoplanin (**a**), lymphatic endothelial hyaluronan receptor-1 (LYVE-1) (**b**) and aminopeptidase P (**c**) on serial sections of normal rat kidney. Podoplanin and LYVE-1-positive lymphatic vessels were rarely detected in the normal architecture of the cortico-medullary tubulo-interstitial area. Lymphatics (* and arrows) were observed adjacent to the intermediate-sized vessels (#). In the glomeruli, podoplanin was detected in podocytes and parietal epithelial cells of Bowman's capsule (**a**). LYVE-1 was also expressed in the glomeruli (**b**). Expression of aminopeptidase P was observed in the peritubular capillaries and glomerular endothelial cells (**c**). Bar = 100 μm. (**d**–**g**) Immunohistochemical staining for podoplanin in the cortex of the unilateral ureteral obstruction (UUO) model. No lymphatic vessels were detected on day 0 (**d**). There were only a few lymphatic vessels in the cortex of the UUO model on day 3 (**e**). On day 9 (**f**) and day 14 (**g**), the number of lymphatic vessels were increased in the tubulo-interstitial injured area. Bar = 200 μm. (**h**–**j**) Lymphatic vessels and blood vessels in the renal medulla in UUO on day 14. Immunohistochemical staining for podoplanin (**h**), LYVE-1 (**i**) and aminopeptidase P (**j**). Lymphatic vessels (*) expressed LYVE-1 and podoplanin, but did not express aminopeptidase P. The entry of the lymphatics (red and blue arrows) was confirmed in the renal pelvis (RP). Vein (#) was positive for aminopeptidase P, but negative for podoplanin and LYVE-1. Bar = 200 μm.

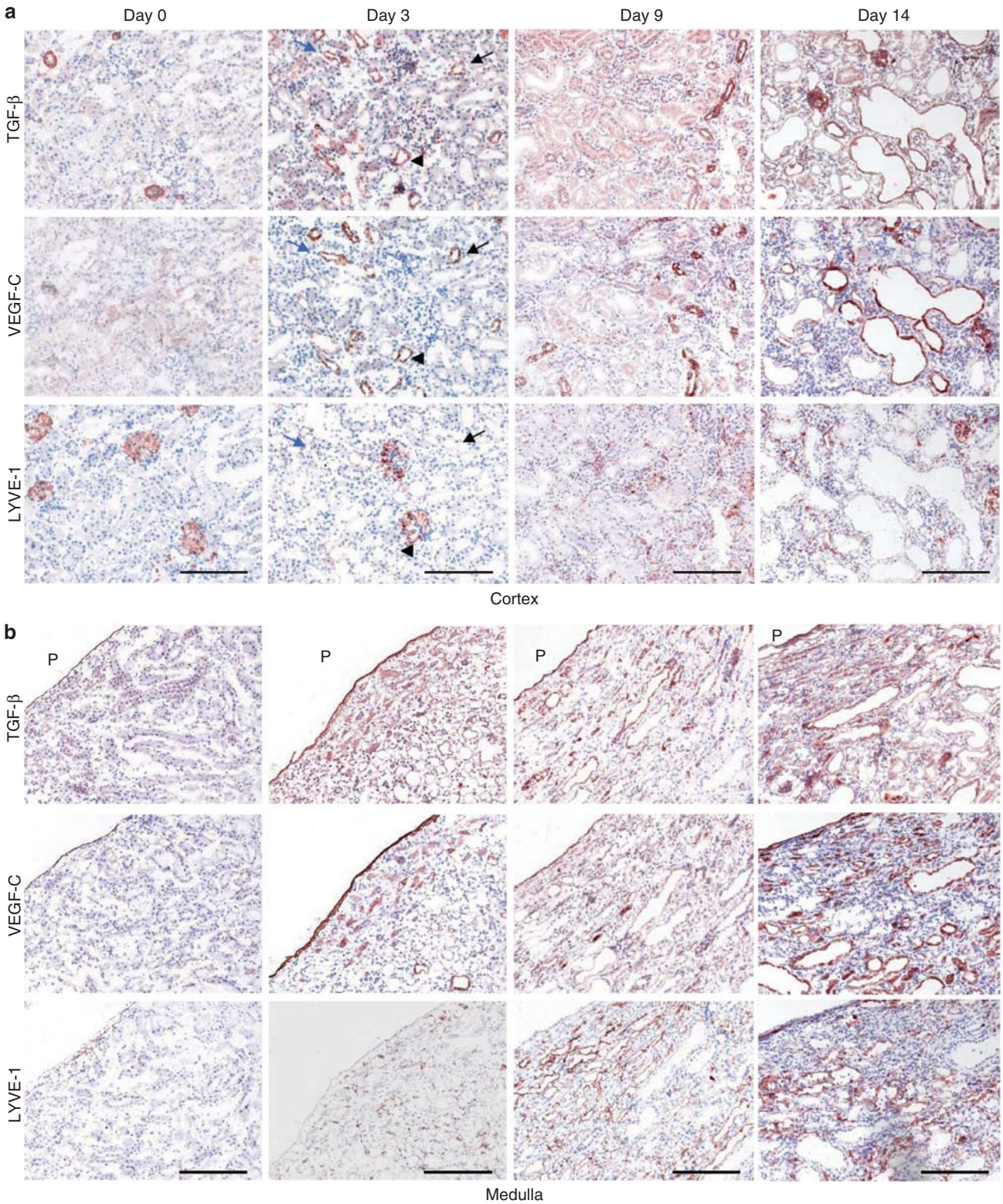

Figure 4 | Expression of growth factors and lymphatic vessels in unilateral ureteral obstruction. Immunohistochemical staining for transforming growth factor-β (TGF-β), vascular endothelial growth factor-C (VEGF-C), and lymphatic endothelial hyaluronan receptor-1 (LYVE-1) on serial frozen sections in the cortex (**a**) and in the medulla (**b**). TGF-β and VEGF-C were expressed in tubular epithelial cells (arrows and arrowheads), and were upregulated with time after ureteral obstruction in both the medulla and cortex. LYVE-1-positive lymphatic vessels were revealed and increased with time. Black and blue arrows and arrowheads indicate the same tubules. Bar = 200 μm. P, renal pelvis.

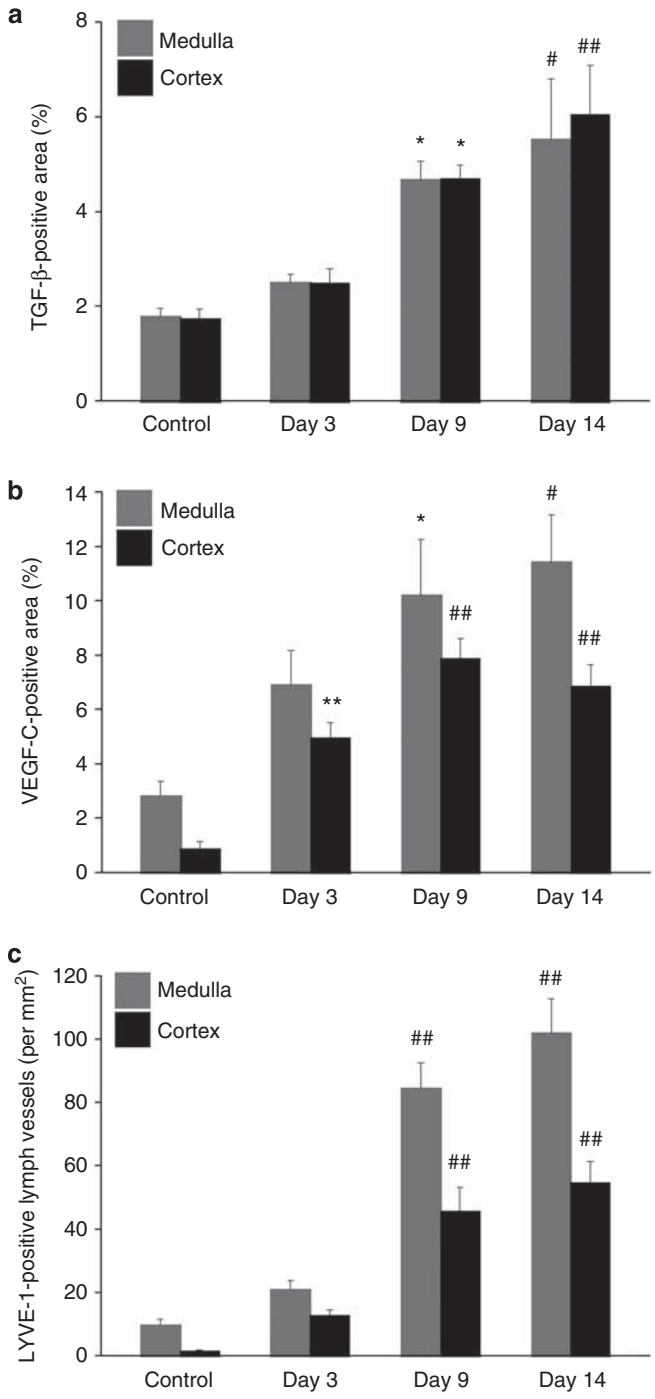

Figure 5 | Quantification of morphological analysis of transforming growth factor-β vascular endothelial growth factor-C (VEGF-C), and lymphatic endothelial hyaluronan receptor-1 (LYVE-1) expression in unilateral ureteral obstruction (UUO) model. TGF-β (**a**) and VEGF-C (**b**) expression increased with time. The number of LYVE-1-positive lymph vessels (**c**) was counted in ten random areas in the medulla and cortex of the UUO kidney. LYVE-1-positive lymphatics increased markedly in the medulla on day 9 and day 14. Values are means ± s.e. ($N = 5$). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{\#}P < 0.01$; $^{\#\#}P < 0.001$ vs. control.

TGF-β1 in these cells was suppressed by TGF-β type I receptor inhibitor LY364947 (LY) in a dose-dependent manner (Figure 9).

On the other hand, in the NRK-49F rat renal fibroblast cell line, VEGF-C mRNA was not upregulated in dose–response experiments (0, 1, 5 or 20 ng/ml) or by 5 ng/ml TGF-β1 at any time points (data not shown).

Effects of TGF-β type I receptor inhibitor (LY364947) on lymphangiogenesis in UUO

To investigate the effects of the TGF-β–VEGF-C pathway on lymphangiogenesis in UUO, we administered LY364947 into the obstructed kidney via the aorta. LY364947 suppressed type III collagen, VEGF-C and LYVE-1 mRNA expression resulting in a reduction in the number of lymphatics (Figure 10).

DISCUSSION

Sustained injury by persistence of the initiating events, such as immunologically mediated glomerulonephritis and tubulo-interstitial nephritis, hemodynamic disorders, and metabolic diseases, may lead to a chronic and fibrogenic inflammatory response involving macrophage infiltration, fibroblast proliferation and accumulation of extracellular matrix.[23–25] Inflammation is thought to have an important role in lymphatic development in several disease conditions, and macrophages have been suggested to stimulate lymphangiogenesis by acting as a source of lymphatic endothelial progenitor cells and by providing a crucial source of pro-lymphangiogenic growth factors.[10,11,13–15,17,18,26,27]

In our recent reports on human renal diseases, we found that lymphangiogenesis was affected by the duration of inflammation and progression of fibrosis, rather than by acute inflammation.[20] Tubulo-interstitial fibrosis in a rat remnant kidney model was also reported to be associated with newly formed lymphatic vessels.[21] In other organs, lymphatic proliferation was confirmed in human idiopathic pulmonary fibrosis and bleomycin-induced pulmonary fibrosis in rats.[18,28] However, conflicting data between tissue fibrosis and lymphangiogenesis have recently been reported.[29–31] TGF-β was reported to directly inhibit growth of lymphatic endothelial cells.[29–31] In addition, TGF-β type I receptor inhibitor enhanced their proliferation.[29] To address the different aspects of lymphatic proliferation in tubulo-interstitial fibrosis on the human biopsy specimens,[20] we investigated the roles of TGF-β and VEGF-C on lymphangiogenesis in UUO. In our analysis of a UUO model on day 3, numerous infiltrating macrophages had already appeared, but only weak upregulation of VEGF-C and slight lymphatic proliferation were confirmed (Figures 2, 5, and 7). From days 9 to 14 in rat UUO, we observed that lymphangiogenesis was associated with tubulo-interstitial fibrosis and chronic inflammation. In addition, VEGF-C expression was elevated in both the cortex and medulla with time after ureteral ligation, and paralleled TGF-β expression (Figures 4, 5, and 7). These findings suggest that the fibrotic process with chronic inflammation may be linked with lymphangiogenesis rather than acute inflammation.

VEGF-C is known to be one of the most important mediators of lymphangiogenesis.[16,32] VEGF-C has been shown to

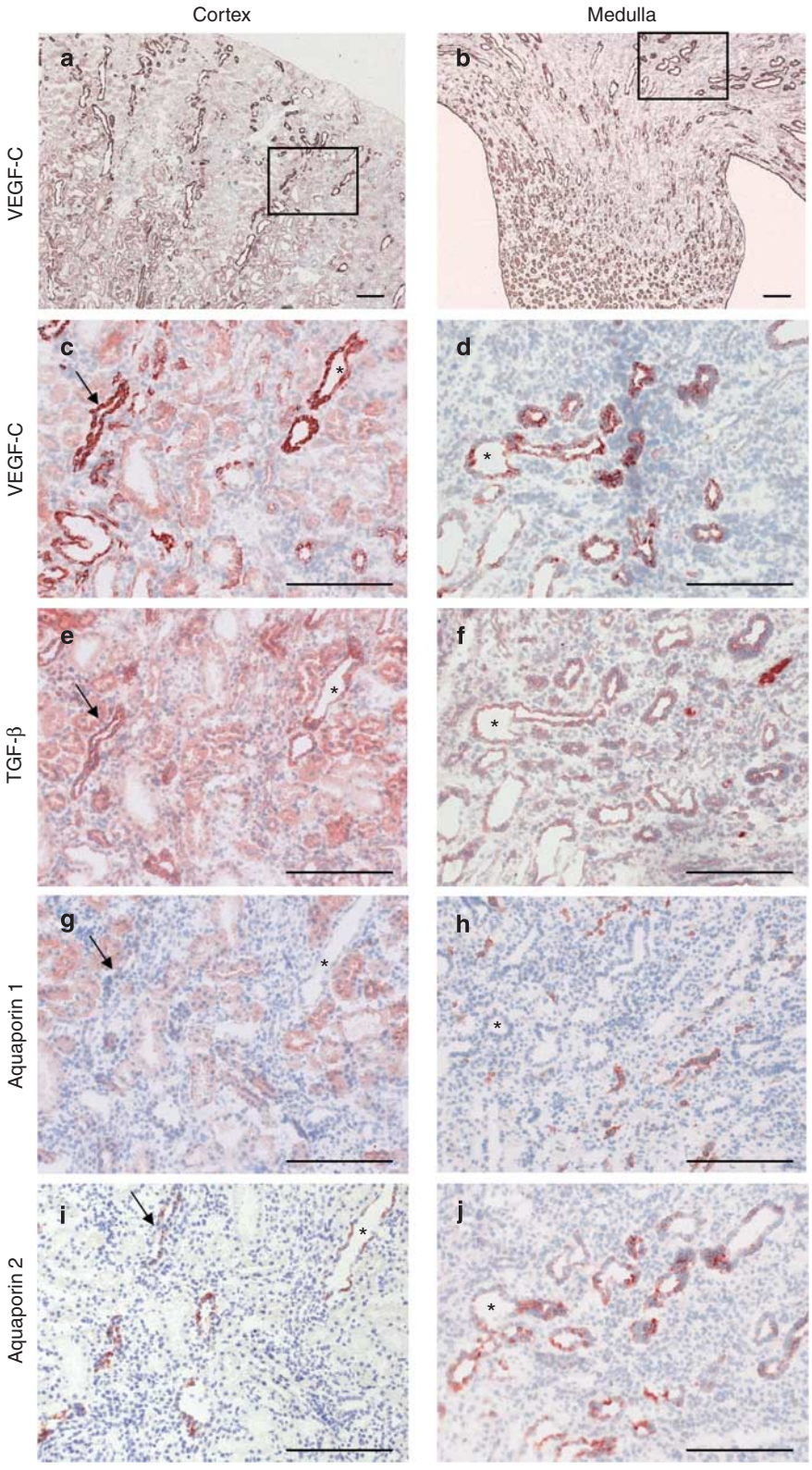

Figure 6 | Localization of vascular endothelial growth factor-C (VEGF-C) and transforming growth factor-β (TGF-β) protein in unilateral ureteral obstruction (UUO) (day 14). Immunohistochemical staining for VEGF-C, TGF-β, aquaporin-1, and aquaporin-2 in the same rat UUO kidney on day 14. VEGF-C is expressed in the aquaporin-1-positive proximal tubules and in the aquaporin-2-positive collecting ducts (arrows and *) in the injured tubulo-interstitial area. TGF-β is often co-localized with VEGF-C both in the cortex and the medulla. (**a**–**d**) VEGF-C staining; (**e**, **f**) TGF-β staining; (**g**, **h**) aquaporin-1 staining; (**i**, **j**) aquaporin-2 staining. Left panel (**a**, **c**, **e**, **g**, **i**): cortex; right panel (**b**, **d**, **f**, **h**, **j**): medulla. (**c**, **d**) Enlargement of the rectangular frame of **a** and **b**, respectively. Bar = 200 μm.

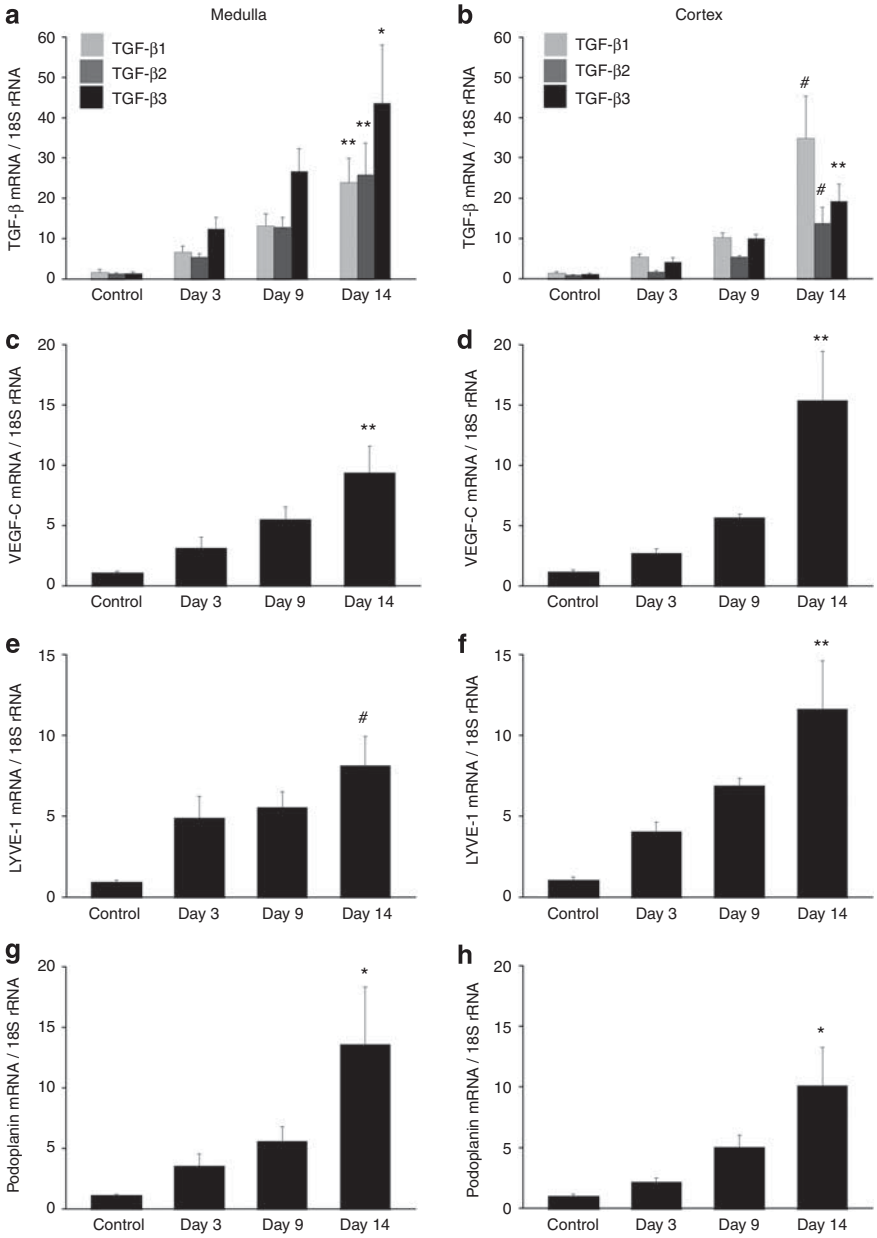

Figure 7 | Quantitative analysis of transforming growth factor-β (TGF-β), vascular endothelial growth factor-C (VEGF-C), lymphatic endothelial hyaluronan receptor-1 (LYVE-1) and podoplanin messenger RNA (mRNA) expression in unilateral ureteral obstruction (UUO) model. Expression of mRNA was analyzed by real-time reverse transcription-polymerase chain reaction. TGF-β, VEGF-C, LYVE-1, and podoplanin mRNA expression in both the medulla and cortex increased and peaked on day 14. (**a**, **c**, **e**, **g**) Medulla of UUO kidney; (**b**, **d**, **f**, **h**) cortex of UUO kidney. (**a**, **b**) TGF-β mRNA; (**c**, **d**) VEGF-C mRNA; (**e**, **f**) LYVE-1 mRNA; (**g**, **h**) podoplanin mRNA. Values are means ± s.e. ($N = 5$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{\#}P < 0.01$ vs. control.

be required for the normal development of lymphatic vessels, particularly those sprouting from the embryonic veins.[3] To clarify the roles of TGF-β in lymphangiogenesis, we investigated VEGF-C induction by TGF-β1 in cultured proximal tubular epithelial cells (HK-2), collecting duct cells (M-1), macrophages (RAW264.7), and fibroblasts (NRK-49F) (Figure 8). We detected basal VEGF-C expression in all cell lines. Notably, significant increases in VEGF-C expression in response to TGF-β1 were seen in cultured proximal tubular epithelial cells, collecting duct cells and macrophages, but not in fibroblasts. In UUO, TGF-β expression increased over time and was preferentially increased in tubular epithelial cells and to a lesser degree in infiltrating macrophages (Figure 4, Supplementary Figures S2–4 online), which is consistent with previous reports of precise studies by *in situ* hybridization, competitive PCR and immunohistochemistry.[33,34] Tubular epithelial cell injury induced by UUO may also affect TGF-β and VEGF-C expression. The strong expression of VEGF-C in aquaporin-1- and -2-positive tubular epithelial cells *in vivo* (Figure 6) and in culture

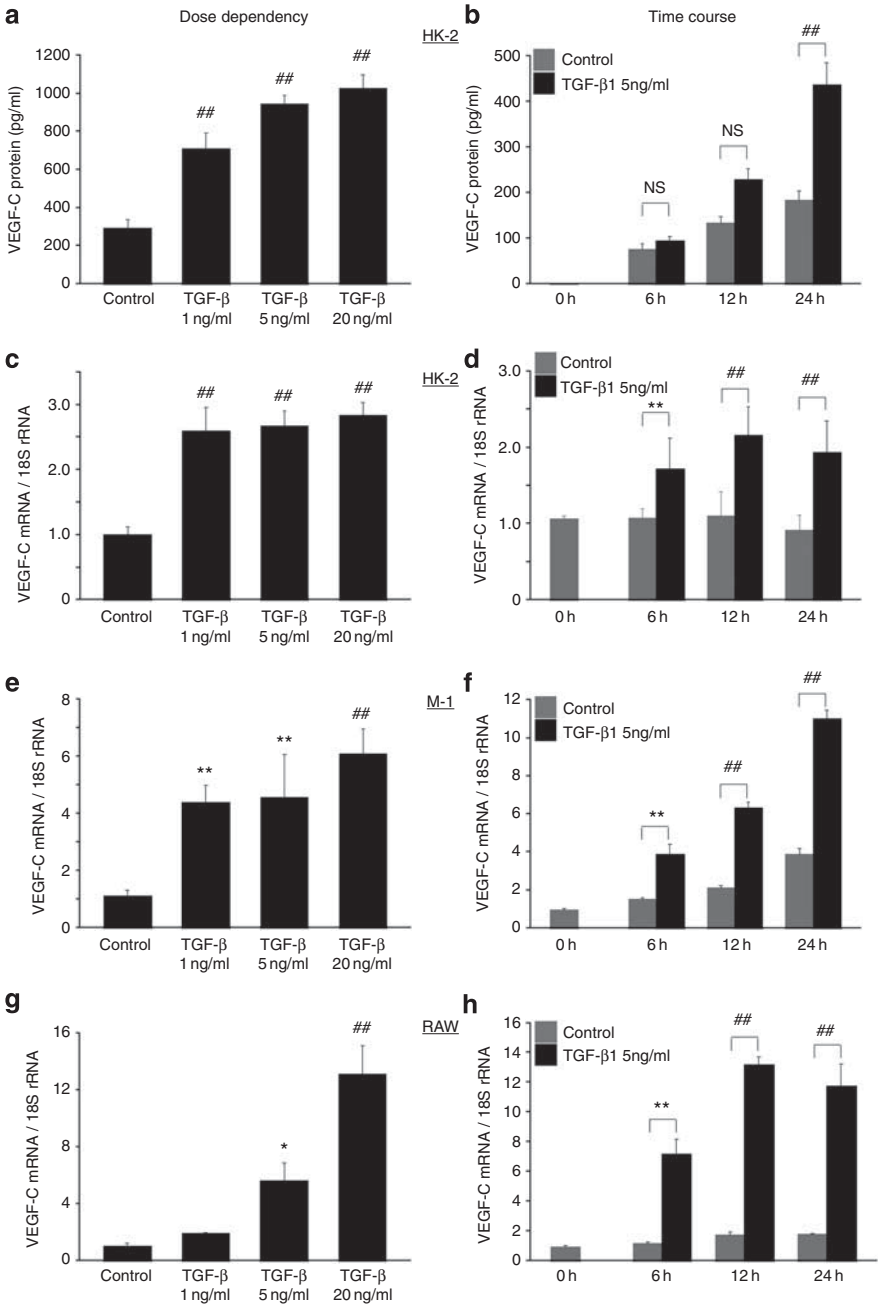

Figure 8 | Vascular endothelial growth factor-C (VEGF-C) expression in cultured proximal tubular epithelial cells (human kidney-2 (HK-2)), collecting duct cells (M-1) and macrophages (RAW 264.7) after treatment with transforming growth factor-β1 (TGF-β1). (**a**–**d**) HK-2 proximal tubular epithelial cell line; (**e**, **f**) M-1 collecting duct cell line; (**g**, **h**) RAW 264.7 macrophage cell line. (**a**–**d**) HK-2 cells, preincubated for 24 h in serum-free medium, were treated with TGF-β1. VEGF-C protein level in the supernatant was determined by enzyme-linked immunosorbent assay (**a**, **b**), and VEGF mRNA level was determined by real-time reverse transcription-polymerase chain reaction (**c**, **d**). 18s ribosomal RNA (rRNA) was used as an internal control. VEGF-C protein levels increased significantly in response to 1, 5, and 20 ng/ml TGF-β1 at 24 h (**a**). VEGF-C mRNA expression was also elevated in response to 1, 5, and 20 ng/ml TGF-β1 at 24 h; it significantly increased at 6 h and peaked at 12 h with 5 ng/ml TGF-β1 (**c**, **d**). (**e**, **f**) M-1 cells were treated with TGF-β1. VEGF-C mRNA expression was significantly increased by stimulation with more than 1 ng/ml TGF-β1. (**g**, **h**) RAW264.7 cells were treated with TGF-β1. VEGF-C mRNA expression was elevated in response to TGF-β1 stimulation. Data are means ± s.e. ($N = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{\#\#}P < 0.001$ vs. control.

studies suggests that tubular epithelial cells are an important source of VEGF-C in UUO (Figure 8). On reverse transcription–PCR, we were not able to detect vascular endothelial growth factor receptor-2 and -3 in cultured proximal tubular epithelial cells, collecting duct cells, fibroblasts, and macrophages (Supplementary Figure S7 online), which suggests that VEGF-C does not act in an autocrine manner. Vascular endothelial growth factor receptor-3 was

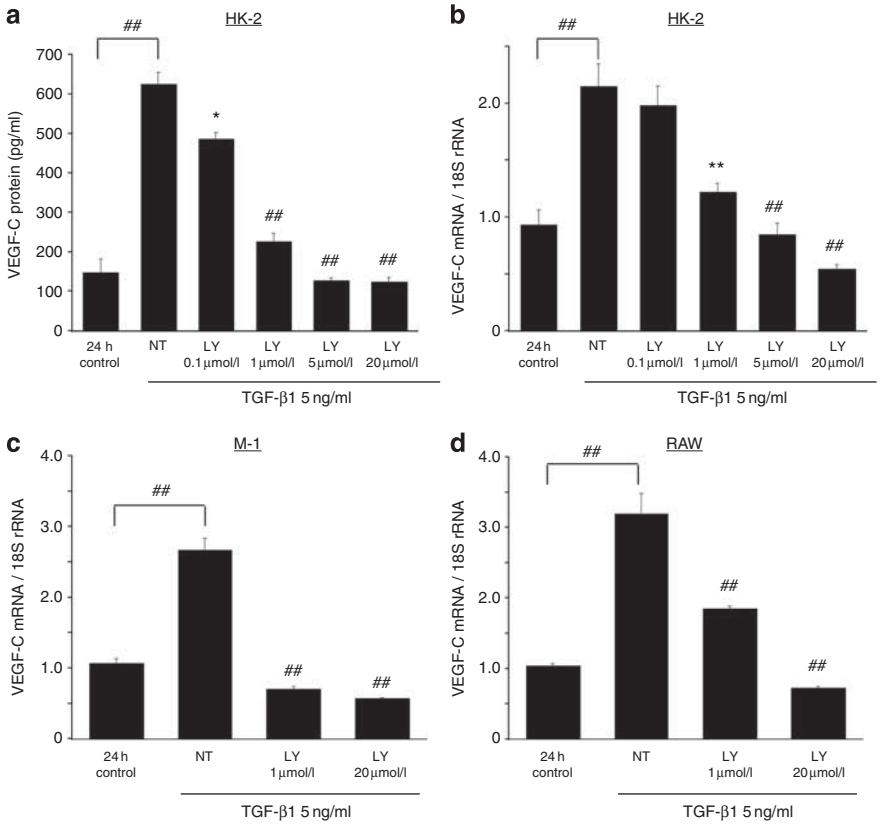

Figure 9 | Inhibition study of vascular endothelial growth factor-C (VEGF-C) expression in cultured proximal tubular epithelial cells (human kidney cells-2 (HK-2)), collecting duct cells (M-1), and macrophages (RAW 264.7) with TGF-β type I receptor inhibitor. (**a**, **b**) HK-2 cells were treated with 5 ng/ml TGF-β1 and TGF-β type I receptor inhibitor LY364947 (LY) for 24 h. VEGF-C protein levels in the supernatant were determined by ELISA. VEGF-C mRNA was analyzed by real-time PCR. VEGF-C protein (**a**) and mRNA (**b**) levels were suppressed by LY in a dose-dependent manner, as compared with non-treatment (NT; TGF-β1 stimulation only) at 24 h. (**c**, **d**) M-1 cells (**c**) and RAW264.7 cells (**d**) were incubated with TGF-β1 (5 ng/ml) and LY. Upregulation of VEGF-C mRNA by TGF-β1 was significantly inhibited by concentrations above 1 μmol/l LY. Data are means ± s.e. ($N = 3$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{\#\#}P < 0.001$ vs. NT.

only detected in the lymphatics *in vivo* (Supplementary Figure S5e–g online). These findings suggest that TGF-β1 is the central inducer of VEGF-C leading to lymphangiogenesis in tubulo-interstitial fibrosis of UUO. In the human chronic glomerular diseases associated with tubulo-interstitial injury, VEGF-C expression was also observed in the tubular epithelial cells.[20] Recent reports have indicated that TGF-β directly inhibits lymphatic endothelial cell proliferation and migration.[29–31] In the cultured NRK-49F fibroblasts of our study, VEGF-C induction by TGF-β1 was not significant, despite the presence of basal expression (Figure 11). These findings suggest that fibroblasts do not directly enhance lymphangiogenesis. However, in the kidney, VEGF-C production by tubular epithelial cells may have a crucial role in the growth of lymphatics, in contrast to the situation in other organs. In the process of lymphatic growth, the effects of VEGF-C produced by these cells may exceed the direct effects by TGF-β on the lymphatic endothelial cells, and the balance between these two factors may control the extent of lymphangiogenesis. The limitation in *in vivo* experiments is the difficulty of systemic administration of TGF-β receptor I inhibitor in UUO. Low doses (1 μg/body g/day intraperitoneally) of LY364947 did not reduce the collagen expression, and rats cannot tolerate high doses (3 μg/body g/day intravenously). Therefore, we administered LY364947 to obstructed kidney via the aorta, which resulted in inhibition of TGF-β, VEGF-C and LYVE-1 (Figure 10). Although it remains unclear whether fibrosis with collagen deposition can modulate lymphangiogenesis, at least TGF-β upregulated VEGF-C in renal cells leading to lymphangiogenesis in UUO. Thus, our data indicate that TGF-β is a critical regulator of VEGF-C, and lymphatic proliferation is likely to depend on the TGF-β–VEGF-C pathway in UUO (Figure 11). Conversely, VEGF-A mRNA expression was not significantly affected (Supplementary Figure S5 online), which is consistent with previous reports. In contrast to the proliferation of lymphatics, peritubular capillary density was shown to decrease in areas of tubulo-interstitial scarring.[35]

In our studies, we used three markers to discriminate between lymphatics and blood vessels. Podoplanin, a transmembrane glycoprotein, is expressed by lymphatic endothelial cells, but not by blood endothelial cells.[10,11] However, it has been reported that podoplanin is expressed in keratinocytes, type I epithelial cells of the lung, mesothelial

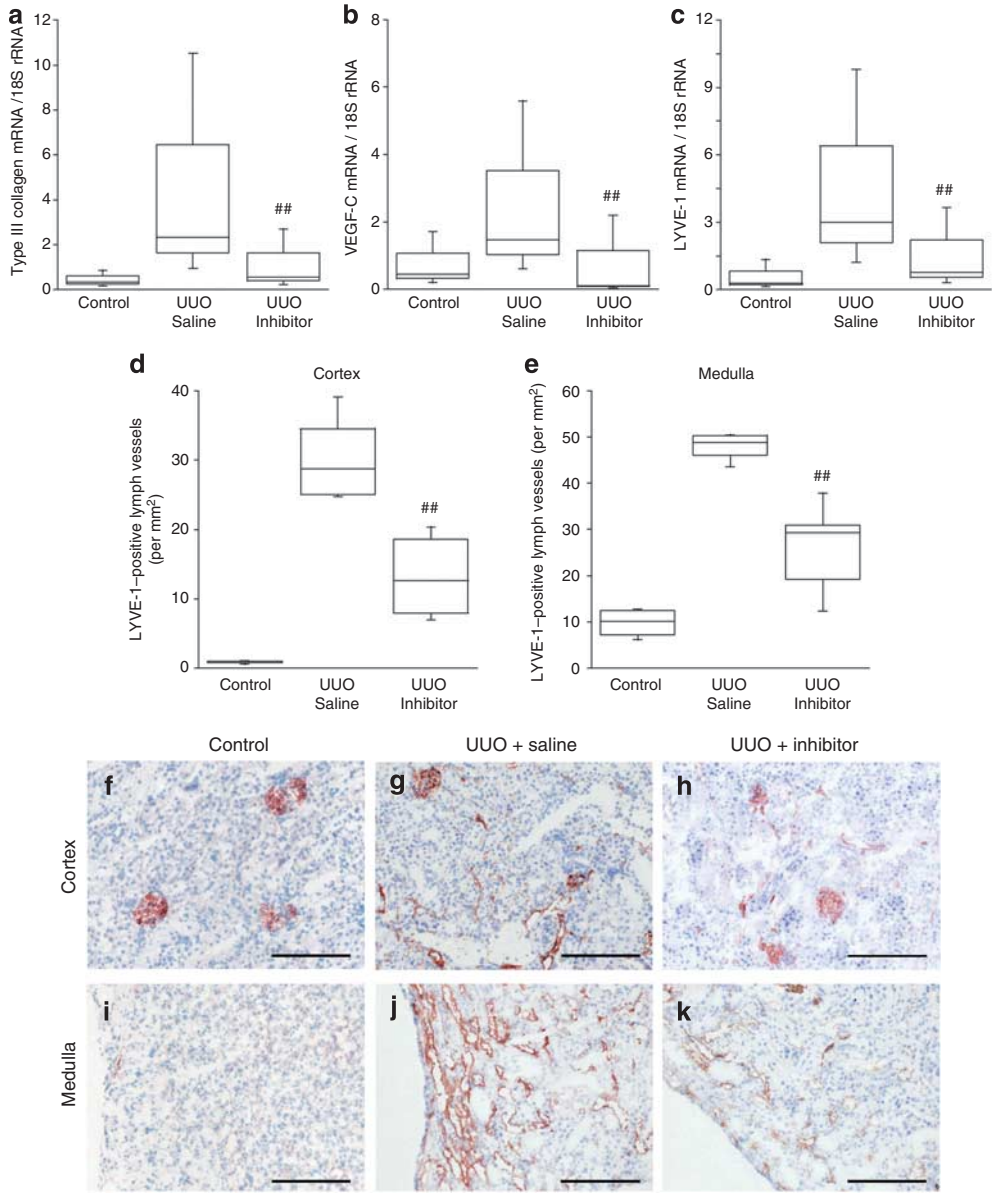

Figure 10 | Effects of transforming growth factor-β type I receptor inhibitor (LY364947) on lymphangiogenesis in unilateral ureteral obstruction (UUO). Type III collagen (**a**), vascular endothelial growth factor-C (VEGF-C) (**b**), and lymphatic endothelial hyaluronan receptor-1 (LYVE-1) (**c**) messenger RNA (mRNA) expression was suppressed by TGF-β type I receptor inhibitor (LY364947) administration via aorta. The number of lymphatics (LYVE-1) was significantly reduced by LY365947 in both the cortex (**d**) and medulla (**e**). Immunohistochemistry of LYVE-1 (**f**–**k**). (**f**–**h**) Cortex; (**i**–**k**) medulla; (**f**, **i**) control kidney; (**g**, **j**) UUO kidney injected with saline; (**h**, **k**) UUO kidney administered LY364947. Data are means ± s.e. ($N = 6$). $^{\#\#}P < 0.001$ vs. UUO injected with saline. Bar = 200 μm. rRNA, ribosomal RNA.

cells, myoepithelial cells, and glomerular podocytes.[21,26] LYVE-1 was first identified as a CD44 homolog and is used as a specific marker of lymphatic endothelial cells.[26,36–38] LYVE-1 is also known to be expressed by liver sinusoids with fenestration, pulmonary capillaries, and some macrophages.[26,39] We found that glomerular endothelial cells, which have a fenestration structure, are extra sites that express LYVE-1 (Figure 3). This was confirmed by double staining with aminopeptidase P, which is a specific marker of endothelial cells in rat blood vessels (Figure 3).[21] In this respect, both markers have limitations as specific markers for lymphatic endothelial cells. We successfully identified the entry of the lymphatic vessels, which were positive for podoplanin and LYVE-1, and negative for aminopeptidase P, into the renal pelvis (Figure 3h–j). A previous study revealed that obstruction of the ureter resulted in a marked increase in thoracic duct lymph flow and pressure in UUO.[40] In addition, lymphatic ligation leads to an increase in renal interstitial pressure.[41,42] In a clinical case of chyluria due to filariasis, occlusion of the thoracic duct was demonstrated by pedal

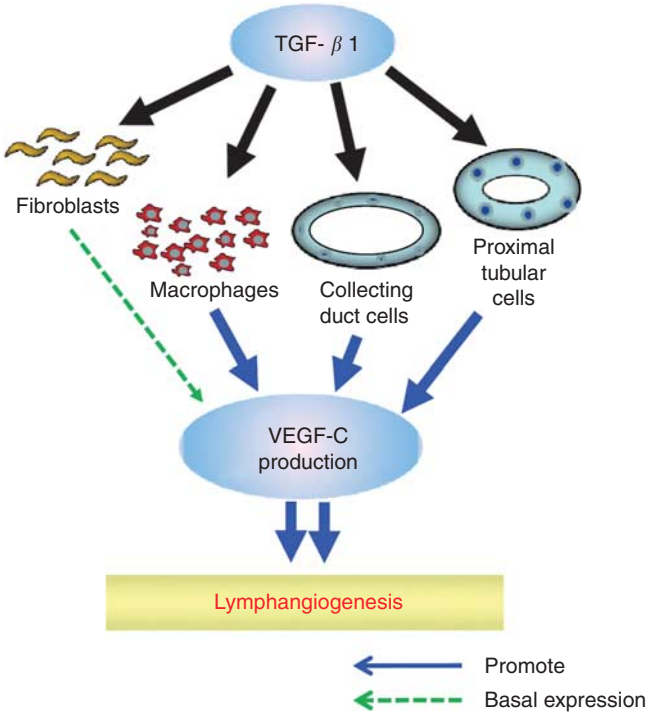

Figure 11 | Possible mechanisms of lymphangiogenesis via the transforming growth factor-β (TGF-β)–vascular endothelial growth factor-C (VEGF-C) pathway in unilateral ureteral obstruction (UUO). TGF-β expression is upregulated in the process of tubulo-interstitial fibrosis in UUO. TGF-β1 expressed by tubular epithelial cells and macrophages induces the upregulation of VEGF-C in proximal tubular epithelial cells (human kidney-2), collecting duct cells (M-1), and macrophages (RAW264.7), leading to lymphangiogenesis. In renal fibroblasts (NRK-49F), VEGF-C was not elevated by TGF-β1, despite basal secretion into the supernatant.

lymphangiography (Supplementary Figure S8a). Enhancement with contrast media on a computed tomography scan of the renal pelvis strongly suggested an interconnection between lymphatics and the urinary excretion system in chyluria (Supplementary Figure S8c online). These findings indicate that there is a connection between pelvic and systemic lymphatic circulation, which may reduce pelvic pressure.

In summary, we demonstrated that lymphatic vessels proliferate and show a correlation with TGF-β expression in UUO. Thus, TGF-β has an important role in the progression of lymphangiogenesis. Tubular epithelial cells and macrophages are involved in the progression of lymphangiogenesis in UUO, and the TGF-β–VEGF-C pathway might have a crucial role. Knowledge regarding the effects of TGF-β on VEGF-C in renal cells may enhance our understanding of the underlying mechanisms and may lead to strategies for modulating lymphatics in chronic renal diseases. Stimulation of lymphatic growth was recently considered as a new target in some disease states. The relative importance of lymphangiogenesis will have to be established by manipulation of VEGF-C levels in chronic renal injury models in the future.

MATERIALS AND METHODS

Animals and experimental design

All animal studies were carried out in accordance with the animal experimentation guidelines of Nagoya University Graduate School of Medicine (Nagoya, Japan). Seven-week-old male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan) initially weighing 210–230 g were used. After induction of anesthesia, the left flank was opened through a small incision and the left ureter was completely ligated with 2-0 suture at two points and cut between the ligatures to prevent retrograde infection. Rats were killed on days 0, 3, 9, and 14 after UUO ($n = 5$ for each time point), and both the obstructed kidney and contralateral kidney were collected. To investigate the TGF-β–VEGF-C pathway on lymphangiogenesis in UUO, we administered TGF-β type I receptor inhibitor LY364947 (2 μg/body g in saline; Calbiochem, La Jolla, CA) or saline alone into the obstructed kidney via the aorta with a 28-gauge needle while clamping the aorta just above the left renal artery on 3 consectutive days from days 2 to 4. Rats were killed and evaluated on day 5. The left kidney was processed for routine histology, immunohistochemistry, and total RNA isolation. Kidney specimens were cut into transverse fragments. One part was fixed for 16 h in 10% buffered formalin and embedded in paraffin by conventional techniques. Sections were stained with periodic acid–Schiff's stain and Masson's trichrome. Formalin-fixed tissue was also used for immunohistochemistry as detailed below. A second part was snap-frozen in liquid nitrogen. Sections (4 μm) were cut with a cryostat and used for immunohistochemistry. A third part of the excised left kidney was cut and divided into the cortex and medulla, which were immersed in RNA*later* (Ambion, Austin, TX) for total RNA isolation. To preserve the integrity and stability of total RNA, all steps were performed at 4 °C under sterile conditions.

Histology and immunohistochemistry

Immunostaining for type III collagen, α-SMA, monocytes/macrophages (ED-1), podoplanin, LYVE-1 and aminopeptidase P was performed on buffered formalin-fixed tissues, and for VEGF-C, TGF-β, and LYVE-1 staining, immunostaining was performed using 4-μm cryostat sections, as described previously (Supplementary Table S1 online).[20,24,43]

Morphological analysis

To assess the relationship between lymphangiogenesis and tubulo-interstitial injury, morphological analysis was separately performed in both the cortex and medulla. Tubulo-interstitial injury was assessed by inflammation (ED-1-positive cells) and by fibrotic processes (α-SMA-positive cells and type III collagen). ED-1-positive macrophages and LYVE-1-positive lymphatic vessels were identified and counted in the cortex and medulla using Zeiss Z1 image microscopy and Axiovision Windows software version 4.4 (Carl Zeiss, Oberkochen, Germany). These were counted in 10 random 750 × 500-μm areas of both the cortex and the medulla at × 200 magnification, and are expressed in terms of counts per square millimeter. Areas positive for α-SMA, type III collagen, TGF-β 1, 2, and 3, and VEGF-C were assessed in 10 random high-powered fields (× 200) of both the cortex and the medulla using MetaMorph 6.3 image analysis software (Universal Imaging, West Chester, PA).

Cell culture study

Human HK-2 proximal tubular cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were

maintained in medium containing Dulbecco's modified Eagle's medium and Ham's F-12 medium (Sigma, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Hyclone, Yokohama, Japan).[44] M-1 cells derived from the mouse cortical collecting duct were purchased from the European Collection of Cell Cultures (ECACC, No. 95092201; Salisbury, UK) and were grown in medium containing a 1:1 mixture of Ham's F-12 medium, Dulbecco's modified Eagle's medium, 5 μmol/l dexamethasone and 5% FBS. RAW 264.7 cells derived from murine macrophages were purchased from ATCC, and were cultured in Dulbecco's modified Eagle's Medium (Sigma) containing 10% FBS. A rat renal fibroblast cell line (NRK-49F) was purchased from ATCC, and was grown in medium containing Dulbecco's modified Eagle's medium supplemented with 5% FBS. All cells were cultured in humidified air with 5% CO_2 at 37 °C. All experiments were performed between passages two and six.

All cells were plated at a density of 2×10^5 cells in 6-cm dishes and grown. Under subconfluent conditions, cells were washed twice with phosphate-buffered saline, and culture medium was replaced with serum-free medium for 24 h to render cells quiescent. Subsequently, cultures were incubated with 5 ng/ml recombinant human TGF-β1 (R&D System, Minneapolis, MN) diluted in serum-free medium. Cells were collected at 0, 6, 12, and 24 h after incubation, and all conditioned media were collected for enzyme-linked immunosorbent assay. For determination of dose response to TGF-β1 stimulation, cells were cultured for 24 h in media supplemented with 0, 1, 5, or 20 ng/ml TGF-β1 after starvation for 24 h. In inhibition studies, cells were incubated with 5 ng/ml TGF-β1 in combination with a selective inhibitor of TGF-β receptor I (LY364947) for 24 h.

Total RNA isolation from rat kidney tissues and cultures cells

Total RNA was extracted from rat kidney tissues and cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA concentrations were estimated using a spectrophotometer (Ultrospec 3300 pro; Amersham Biosciences, Tokyo, Japan). Rat kidney tissues were immersed in RNA*later* (Ambion, Austin, TX) for 1 day. The mixture was ground for 2 min with 5-mm tungsten carbide beads at a frequency of 27 Hz using a mixer-mill grinder according to the manufacturer's instructions (Tissuelyser; Qiagen).

Polymerase chain reaction

For both animal samples and cultured cells, first-strand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) for animal samples or High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) for cultured cells, according to the manufacturers' instructions. Total RNA (1 μg) was then reverse transcribed. Real-time PCR analysis was performed with an Applied Biosystems Prism 7500HT sequence detection system using TaqMan gene expression assays, as described previously.[45] TaqMan gene expression assays (Applied Biosystems) were used (Supplementary Table S2 online). 18S ribosomal RNA was used as an endogenous control.

VEGF-C enzyme-linked immunosorbent assay

Secretion of VEGF-C into culture supernatants was determined using the Human VEGF-C Assay kit (IBL, Takasaki, Japan), according to the manufacturer's instructions.

Statistical analysis

Values are expressed as means ± s.e. Comparisons among groups were performed by one-way analysis of variance followed by Dunnett's or Tukey's HSD (honestly significant difference) multiple comparison test. Comparisons between LY364947 and saline groups in *in vivo* experiments were assessed by Mann–Whitney *U*-test. Differences were considered to be statistically significant at $P<0.05$. All analyses were performed using SPSS software (SPSS, Chicago, IL).

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

Grant numbers and sources of support: This work was supported by a Grant-in-Aid for Scientific Research from the Ministry Education, Science, and Culture, Japan (# 20590972), and a 2008 research grant from the Aichi Kidney Foundation, Japan. Financial supports: none. We are grateful for the technical assistance of Mr Norihiko Suzuki, Ms Keiko Higashide, Ms Naoko Asano and Ms Yuriko Sawa (Department of Nephrology, Nagoya University, Nagoya, Japan).

SUPPLEMENTARY MATERIAL

Table S1. List of the antibodies.
Table S2. Primers for real-time PCR (TaqMan Gene Expression Assays).
Figure S1. Double-immunofluorescence staining for aminopeptidase P and LYVE-1 in normal glomeruli.
Figure S2. Expression of TGF-β, VEGF-C and ED-1 positive macrophages in UUO on day 3.
Figure S3. Double staining for TGF-β and VEGF-C on the frozen section in UUO on day 14.
Figure S4. VEGF-C was expressed by ED-1 positive macrophages in UUO on day 9.
Figure S5. Expression of VEGFR3 in UUO.
Figure S6. Quantitative analysis of VEGF-A mRNA expression in UUO model.
Figure S7. Expression of VEGF-receptor 2 and 3 mRNA in cultured proximal tubular epithelial cells (HK-2), collecting duct cells (M-1) macrophages (RAW 264.7) and fibroblasts (NRK-49F).
Figure S8. Lymphangiography in a case with chyluria due to filariasis.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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