Studies on the Roles of Angiogenic Factors in the Glomeruli on the Progression and Recovery of Anti-Thy-1 Nephritis

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井上　薫

Studies on the roles of angiogenic factors in the glomeruli on the progression and recovery of anti-Thy-1 nephritis.

(抗 Thy-1 腎炎の進行・寛解における糸球体での
血管新生因子の役割に関する研究)

井上　薫

Contents

Introduction

Glomerulonephritis is very important disease of the nephropathies in human and animals. The mechanisms of the development and progression of this disease are complicated and extensive studies have been performed from the various viewpoints. Many studies have been performed *in vitro* and *in vivo* until recent years, and it has been revealed that many cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) are involved in the progression of glomerulonephritis in experimental models and spontaneous human diseases (Yoshimura, *et al.* 1991., Iida, *et al.* 1991., Coimbra, *et al.* 1991., Yoshioka, *et al.* 1993.). These studies performed from the viewpoint to reveal the role of the cytokines in the progressive process of glomerulonephritis such as mesangial proliferation and glomerular sclerosis. For example, it has been shown that PDGF is produced and secreted from the glomerular mesangial cells and is involved in mesangial cell proliferation in the autocrine/paracrine manner *in vitro* and *in vivo* (Yoshimura, *et al.* 1991., Iida, *et al.* 1991.). These studies would lead to the resolution of the progressive mechanisms of this disease and the establishment of new therapeutic strategies.

The anti-Thy-1 nephritis has been studied as the model of mesangioproliferative nephritis. This model is induced by a single intravenous injection of either anti-thymocyte antibody or complement fixing monoclonal or polyclonal antibody to the Thy-1 antigen which is localized on the surface of mesangial cells in rats (Yamamoto, *et al.* 1986., Yamamoto, *et al.* 1987., Bagchus, *et al.* 1986., Johnson, *et al.* 1990.). After injection of anti-Thy-1 antibody into rats, the loss of mesangial cells with disruption of the mesangial

matrix ("mesangiolysis"), which is followed by massive mesangial cell proliferation with phenotypic change of these cells and the accumulation of extracellular matrix, is observed (Yamamoto, *et al.* 1987., Bagchus, *et al.* 1986., Johnson, *et al.* 1990. Johnson, *et al.* 1991., Floege, *et al.* 1991.). This model has often been used to study the mechanism of mesangial proliferation and matrix accumulation and the role of cytokines in these processes. However, in many experimental models, this model could be useful for the study of the recovery mechanism of injured glomeruli because these lesions quickly subside and this experimental model also has been recognized as a reversible model of glomerulonephritis.

Recently, the recovery of the glomerular diseases has been noticed and a "resolution" phase of anti-Thy-1 model has been studied to understand the mechanisms of spontaneous glomerular capillary repair after the disruption of glomerular structure. One of the angiogenic factors, vascular endothelial growth factor (VEGF) seems to be the representative factor, which might be involved in the reconstruction of the injured glomeruli. VEGF and its specific receptors, flt-1 and flk-1, mediate endothelial cell proliferation and increase of vascular permeability (Ferrara, 1999). In the renal glomeruli, it has been reported that VEGF is produced by the podocytes, activated mesangial cells, infiltrating leukocytes in normal and diseased kidneys (Brown, *et al.* 1992., Gruden, *et al.* 1997., Iijima, *et al.* 1993., Noguchi, *et al.* 1998., Shulman, *et al.* 1996., Thomas, *et al.* 2001.) and endothelial cells *in vitro* (Uchida, *et al.* 1994.). The receptor flk-1 is expressed in normal, damaged and regenerating glomerular endothelial cells (Masuda, *et al.* 2001.) as well as mesangial cells (Thomas, *et al.* 2000.). Recent experimental studies indicated that the

glomerular endothelial cells were active participants in the capillary repair and restoration of glomerular architecture. It was also indicated that VEGF and FGF-2 played an important role in endothelial cell proliferation and capillary repair in damaged glomeruli in anti-Thy-1 nephritis (Masuda, *et al.* 2001., Iruela-Arispe, *et al.* 1995., Ostendorf, *et al.* 1999.). However, suitable method has not been used as regards the quantitative analysis of local VEGF and flk-1 mRNA expression in the glomeruli of anti-Thy-1 nephritis.

It seems to be very important to analyze gene or protein expression of the cytokines in the glomeruli for the studies of the mechanism of the glomerular diseases. To extract RNA or protein for the analysis of these expressions in the glomeruli, glomeruli have been obtained by sieving method in which the glomeruli could be isolated from the renal cortex using stainless steel meshes. Moreover, recent development of laser microdissection (LMD) system and laser capture microdissection (LCM) system enables us to obtain single cells or target tissues from the tissue sections using a laser beam, and some studies have shown that it is possible to analyze expression of the cytokines in the glomeruli using these techniques (Kohda, *et al.* 2000).

The author first suspected that VEGF might contribute to the reconstruction of damaged glomeruli by stimulating the proliferation of glomerular cells including glomerular endothelial cells as well as mesangial cells. Therefore, the author performed a series of the studies to understand the role of VEGF and flk-1 in the glomeruli of anti-Thy-1 nephritis using the techniques to analyze local gene expression of VEGF and flk-1. In Chapter Ⅰ, to see the best method for glomerular isolation to evaluate gene expression in the glomeruli, the sieving method, LCM and LMD systems were performed

and compared. In Chapter Ⅱ, to establish the method for the detection of the local mRNA expression of VEGF and flk-1 by RT-PCR in the renal glomeruli, LMD system which was confirmed to be the best method for glomerular isolation in Chapter Ⅰ, was applied to normal rat kidneys. And then the usefulness of this system for the application of the research for glomerular diseases was discussed. In ChapterⅢ, the detailed quantitative evaluation of VEGF and flk-1 was performed using LMD system to clarify the role of VEGF and flk-1 in the glomeruli of anti-Thy-1 nephritis. In ChapterⅣ, the author noted FGF-2 and its specific receptor, FGFR as an another candidate to play an important role in glomerular recovery, and studied the role of FGF-2 and FGFR in the glomeruli of anti-Thy-1 nephritis by the same methods as ChapterⅢwith respect to VEGF.

A part of contents in Chapter Ⅱ has been published in the following paper.

Inoue, K., Sakurada, Y., Murakami, M., Shirota, M. and Shirota, K. 2003. Detection of gene expression of vascular endothelial growth factor and flk-1 in the renal glomeruli of the normal rat kidney using the laser microdissection system. *Virchows Arch.* **442**: 159-162.

Chapter I

The methodological investigation for isolation of the renal glomeruli to evaluate local gene expression

Introduction

Glomerulonephritis is very important disease among human and animal nephropathies, and many cytokines such as platelet derived growth factor(PDGF), transforming growth factor- β (TGF- β) or vascular endothelial growth factor(VEGF), are involved in the mechanisms of progression and recovery of this disease (Yoshimura, *et al.* 1991., Iida, *et al.* 1991., Coimbra, *et al.* 1991., Yoshioka, *et al.* 1993., Thomas, *et al.* 2000.). Analyzing the expression of these factors in the glomeruli is very important to understand the pathogenesis, and to provide new approaches to the therapy of glomerulonephritis.

In the previous studies of glomerulonephritis, RNA extracted from renal cortex has been used to analyze gene expression of the cytokines. However, the sample might contain RNA derived from other component of kidney such as tubules, connective tissue or infiltrating cells in the interstitium and it is impossible to analyze gene and protein expression in the glomeruli exactly. The sieving method, which can isolate glomeruli from the renal cortex with stainless steel meshes, has been used to obtain the renal glomeruli for the study of local gene and protein analysis (Masuda, *et al.* 2001). Moreover, the recent development of the laser microdissection (LMD) or laser capture microdissection (LCM) system, which can dissect target cells or tissues from tissue sections using laser, enables us to collect renal glomeruli more easily and correctly.

In this chapter, we compared these three methods to find the best method to collect glomeruli and analyze gene expression in the glomeruli efficiently and exactly.

Materials and methods

Sieving method (Fig.1a)

Eleven normal male CRJ/CD rats (Charles River Japan, Inc., Yokohama, Japan), age between 8 and 31 weeks, were sacrificed by bleeding under anesthesia and their kidneys were collected. Almost all the renal cortex from each animal was collected and immediately used for the three-stage sieving method under RNase free condition. Briefly, three different size of stainless steel meshes (the mesh size were 180, 125 and 75 μm) were used to sieve the renal cortex and at the last stage of sieving, renal glomeruli were obtained on the 75 μm mesh (Fig.1a①). The isolated glomeruli were collected into the 50ml centrifuge tubes after flushing the mesh from the back side using RNase-free PBS (Fig.1a②) and centrifuged for 10 min at 6,500rpm at 4℃ to collect them to the bottom of the tubes (Fig.1a③). After discarding the supernatant fluid (Fig.1a④), the smear sample of isolated glomeruli were made and stained with hematoxylin-eosin (H.E.) to confirm the quality of the samples (Fig.1a⑤). Total RNA was extracted from isolated glomeruli using TRIzol reagent (Life Technologies, Rockville, MD, USA.) according to the manufacturer's instructions, using Ethachinmate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (Fig.1a⑥,⑦). First-strand cDNA was made from total RNA using the SuperScript First-strand Synthesis System for reverse transcriptase (RT)-PCR (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) with oligo $(dT)_{12\text{-}18}$ primers. PCR was performed with a TaKaRa PCR Thermal Cycler MP (TaKaRa Shuzo, Kyoto, Japan) with oligonucleotide primers as follows: GAPDH (sense: 5'-TCCCTCAAGATTGTCAGCAA-3'

and antisense: 5'-AGATCCACAACGGATACATT-3') (Terada, *et al.* 1992.). The PCR reaction was performed with TaKaRa *Taq*™ (TaKaRa Shuzo, Kyoto, Japan) under the following conditions: denature at 94℃ for 5 minutes; and then 40 cycles of denature at 94℃ for 1 min; annealing at 55℃ for 1 min; extension at 72℃ for 2 min; and finally extension at 72℃ for 7 minutes. Four microliters of the PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide and the amplified products were verified to be a single band of 308bp in size. Total time until putting the isolated glomeruli into TRIzol regent after dissection of the kidney from the rat was about 10 to 15 min.

Laser capture microdissection (LCM) (Fig.1b)

Three normal male Fischer 344 rats (Charles River Japan, Inc., Yokohama, Japan), age 8 weeks, were sacrificed by bleeding under anesthesia and their kidneys were collected. A part of the renal cortex was quickly frozen in liquid nitrogen and stored at -80℃ until use. Frozen sections (10 μm) of the renal cortex were made and mounted on glass slides sterilized at 250℃ for 30 min (Fig.1b①). They were fixed with 70% ethanol at -20℃ for 1 min, gently washed serially with diethylpyrocarbonate (DEPC)-treated water, 70%, 90%, 99% ethanol and xylen at room temperature for 1 min and finally soaked in xylen at room temperature for 10 min (Fig.1b②). The sections were air-dried (Fig.1b③) and stored at -80℃ until use. The sections were covered with transfer film on the tube cap and the glomeruli were collected on the films after exposure of brief IR laser pulses which were applied as 15 μm diameter circle to the sections using the LCM system, LM200 (Olympus

corporation, Tokyo, Japan) (Fig.1b④). Fifty to eighty glomeruli were obtained on a film (Fig.1b⑤) and the tube caps with the films or the films themselves were collected to the 0.5ml tubes filled with 200μl of TRIzol regent. After 30, 300, 400, 800 and 1000 glomeruli were collected in each tubes, they were incubated at room temperature, mixed sufficiently with vortex or sonicated (Fig.1b⑥), and total RNA was extracted according to the manufacturer's instructions using Ethachinmate (Fig.1b⑦). The following experiment was performed in the same way described in sieving method. For RT-PCR, SUPERSCRIPT™ One-step RT-PCR with PLATINUM Taq (Life Technologies) was used in some cases according to the manufacturer's instructions.

Laser Microdissection (LMD) (Fig.1c)

Two normal 8-week-old male Wistar rats (Charles River Japan, Inc., Yokohama, Japan) were sacrificed by bleeding under anesthesia and their kidneys were collected. A part of the renal cortex was quickly frozen in liquid nitrogen and stored at -80℃ until use. Frozen sections (5 μm) of the renal cortex collected from a normal Fisher rat described previously were made and mounted on glass slides covered with PEN foil (2.5 μm-thick) (Leica Microsystems, Wetzlar, Germany) (Fig.1c①). They were fixed with 70% ethanol at -20℃ for 10 sec, gently washed twice with diethylpyrocarbonate (DEPC)-treated water (Fig.1c②), and thoroughly air-dried (Fig.1c③). The sections were then stained with 0.05% toluidine blue (TB) solution, pH 4.1, (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 seconds (Fig.1c ④), and the TB solution was rinsed out with DEPC-treated water, and the

sections were air-dried. The renal glomeruli were dissected from the frozen sections with the LMD system using a 337nm- nitrogen ultraviolet (UV) laser (Leica Laser Microdissection System, Leica Microsystems, Wetzlar, Germany) (Fig.1c⑤). The glomeruli were dissected from a section and dropped immediately into a microcentrifuge tube cap filled with 30 μl of TRIzol reagent (Fig.1c⑥) and total RNA was extracted using Ethachinmate (Fig.1c⑦). RT reaction was performed with SUPERSCRIPT™ One-step RT-PCR with PLATINUM Taq according to the manufacturer's instructions. PCR and electrophoresis were performed in the same way as described in sieving method.

Results

Sieving Method

This method needed a skillful assistant to perform speedily. Many glomeruli could be collected, however the samples often contained other renal component such as tubules (Fig. 2a). Moreover, quantity of total RNA obtained was not stable (2.31 to 155.79 μ g) and little RNA could be obtained in some samples despite many glomeruli could be isolated. GAPDH could be amplified by PCR using cDNA derived from 2 μ g of total RNA and its specific size band (308bp) was observed by electrophoresis (Fig. 2b).

LCM

By this method, 30 to 1000 glomeruli were collected from non-stained frozen sections. The glomeruli were easily observed and distinguished from other renal component in the sections on the monitor of the LCM system. Several spots of laser pulse were needed to capture one glomerulus and the laser was irradiated directly to the glomeruli. The collected glomeruli adhered on the films did not take away easily from the films and could be observed in TRIzol regent even after vortex or sonication. After ethanol precipitation, RNA was rarely observed in ethanol, and GAPDH was not amplified by RT-PCR although 1000 glomeruli were collected (Fig.3a). However, GAPDH gene expression in the 800 glomeruli was detected only once when ultrasonic wave treatment and the regent for one-step RT-PCR were used (Fig.3b).

LMD

Fifty glomeruli were collected from frozen sections stained with toluidine blue solution and they could be distinguished easily from other renal component (Fig.4a). Because an UV laser was irradiated along the line, which was traced around each glomerulus by computer mouse, the renal glomeruli were dissected exactly and were not irradiated directly to them (Fig.4b). Total RNA could be immediately extracted from the dissected glomeruli in the TRIzol regent filling in a tube cap in this system. After ethanol precipitation, RNA could be observed at the bottom of the tube and GAPDH was amplified by PCR using cDNA derived from total RNA of 50 glomeruli (Fig.4c).

Discussion

In this chapter, three different methods for isolation of the glomeruli were performed and compared their validity for analyzing local gene expression in the glomeruli.

Many glomeruli could be collected in a short time by sieving method. However, tubules often contaminated glomerular samples and quantity of total RNA was inconstant and some samples could not be applied for PCR because sufficient total RNA could not be obtained. These data suggest that this method is not suitable for the analysis of gene expression in the glomeruli.

LCM and LMD systems have been recently developed and these systems enable us to obtain target cells or tissues from the tissue sections using laser. These systems have some differences such as the type of laser, the way of laser application and collecting the dissected samples (Fig. 1). As regards

collecting glomerulus, LCM needed several spots of IR laser and the laser was irradiated directly on the glomeruli, while the UV laser was not irradiated directly on the glomeruli, but was irradiated along the line traced around the glomerulus by LMD system, which resulted in little injury. And the collected glomeruli firmly adhered to the films and it took time to dissect 50 to 80 glomeruli before they were applied in the reagent for RNA extraction in the LCM system, while dissected glomeruli dropped immediately into the tube cap filled with the regent for RNA extraction in the LMD system. These results suggest that LMD system is better than LCM system on the point of collecting glomeruli in the tubes.

In the LCM system, the collected glomeruli were adhered firmly on the films and were not easily taken away from the films by vortex or sonication. This might affect the RNA extraction and the results of RT-PCR analysis. Moreover, the results of PCR had little reproducibility in this study. On the other hand, total RNA of the collected glomeruli was immediately extracted after the dissection by LMD system and in this study, only 50 glomeruli were enough to detect GAPDH gene expression by RT-PCR. These results also suggest that LMD system is better than LCM system to analyze gene expression in the glomeruli.

Some previous studies using LCM or LMD system suggested that the preparation of the tissue sections including fixation and staining influenced the result of gene analysis (Goldworthy, *et al.* 1999., Murakami, *et al.* 2000.). And it was indicated that the selection of the regents for RNA extraction and RT reaction related to the sensitivity of RT-PCR (Boylan, *et al.* 2001.) The microdissection techniques have been applied for human and rat renal tissues,

and it was indicated that 10 and 30 glomeruli dissected using LCM or laser-manipulated microdissection (LMM) and laser pressure catapulting (LPC) could be used to analyze gene expression by RT-PCR and real-time PCR (Nagasawa, *et al.* 2000., Kohda, *et al.* 2000.). In this study, frozen sections fixed in 70% ethanol were prepared and stained with toluidine blue solution followed by dissection using LMD system. Total RNA derived from dissected 50 glomeruli was extracted and applied to RT-PCR, and GAPDH mRNA in the glomeruli could be successfully detected. Further evaluation for the methods including the thickness of the frozen sections, the number of the glomeruli and regents for RNA extraction would improve the usefulness of the LMD system in the study of glomerular diseases.

The microdissection techniques enable us to analyze the relationship between morphological change and local gene expression and it was reported that target cells detected by immunostaining could be dissected using this technique (Fink, *et al.* 2000.). In this chapter, the author concludes that LMD system is the best method to analyze gene expression in the renal glomeruli. The application of this system might be very useful for the studies of glomerulonephritis and the combination of LMD system and real-time PCR would contribute to the resolution of the mechanisms of the progress and recovery of the disease.

Abstract

Glomerulonephritis is very important disease and many cytokines such as platelet derived growth factor (PDGF), transforming growth factor (TGF-β) or vascular endothelial growth factor (VEGF) are involved in the mechanisms of progression and recovery of this disease. Analyzing the expression of these factors in the glomeruli is very important to understand the pathogenesis and to provide new approaches to the therapy of glomerulonephritis. To isolate renal glomeruli for the analysis of gene expression, some methods including sieving method have been used, and recently, laser microdissection (LMD) or laser capture microdissection (LCM) system which enable us to dissect target tissue such as glomeruli from the tissue sections have developed. In this chapter, these three different methods (sieving method, LMD and LCM system) were compared to find the best method to isolate glomeruli for the analysis of gene expression in the glomeruli efficiently and exactly. At first, sieving method was performed using three different sizes of stainless steel meshes and glomeruli were isolated from the renal cortex of normal male CRJ/CD rats. Second, frozen sections of the renal cortex derived from normal male Fischer 344 rats were made and after fixation and washing, they were air-dried. And then, the glomeruli were dissected from their sections and collected to the transfer films on the tube cap using LCM system. Third, frozen sections of the renal cortex derived from normal male Wistar rats and after fixation, staining and washing, they were air-dried. And then glomeruli were dissected from their sections and dropped into the tube cap filled with RNA extraction regent using LMD system. After confirming the quality of the samples, total RNA was extracted from the collected glomeruli and analyzed

their concentration, and RT-PCR was performed to detect GAPDH gene expression in the glomeruli. The glomerular samples obtained by sieving method contained other renal component and quantity of total RNA obtained was not stable. With sieving method, GAPDH could be amplified and confirmed its specific size band by electrophoresis. It was possible to dissect glomeruli exactly and to collect them easily from the frozen sections using LMD and LCM systems. However, it was difficult to take the collected glomeruli away from the films and to amplified GAPDH gene by PCR although 1000 glomeruli were collected using LCM system, while dissected 50 glomeruli using LMD system were collected efficiently and GAPDH gene expression was easily detected. In conclusion, LMD system is the best method to analyze gene expression in the renal glomeruli. The combination of this system and other technique such as real-time PCR would contribute to the resolution of the mechanisms of the disease.

Figure Legends

Fig.1: Flowchart of three methods for glomerular isolation. (a) Sieving method, (b) Laser capture microdissection (LCM) and (c) Laser microdissection (LMD) technique.

Fig.2: The quality of the glomerular sample obtained by sieving method and GAPDH gene expression.

(a) The micrograph of smear sample stained with hematoxylin-eosin (H. E.). The glomerular samples often contained other renal component such as tubules (arrow). (b) GAPDH gene expression was amplified by RT-PCR and confirmed its specific size band (308bp) by electrophoresis. Lane1: molecular weight marker, lane 2 to 10: cDNA samples derived from isolated glomeruli.

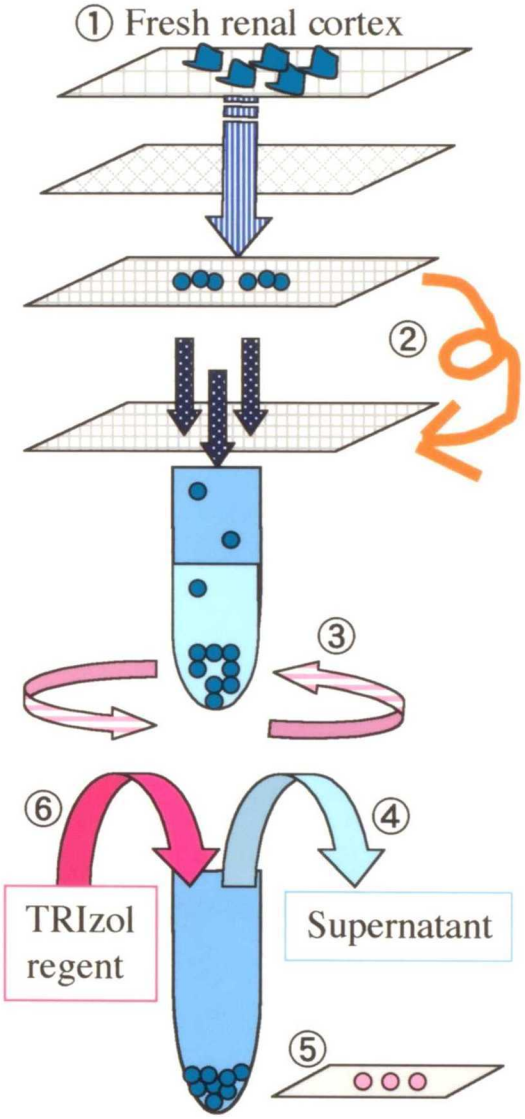
Fig. 3: Detection of GAPDH gene in the glomeruli isolated using LCM system.

It was impossible to detect GAPDH gene expression although 1000 glomeruli were collected using LCM system (a). However GAPDH gene expression could be detected only once using cDNA derived from 800 glomeruli collected by same system. (a) Lane 1: molecular weight marker, lane 2: cDNA sample derived from 1000 glomeruli, lane 3: cDNA sample derived from renal cortex of normal rat, lane 4,5: negative control (no template). (b) Lane 1: molecular weight marker, lane 2, 3: cDNA sample derived from 800 glomeruli.

Fig. 4: The condition of the frozen section and GAPDH gene expression in the glomeruli collected by LMD system.

The glomeruli could be observed easily and distinguished from other renal component in the frozen sections stained with toluidine blue (TB) solution (a). The glomeruli were dissected exactly by the UV laser, which was irradiated along the line, traced around the glomerulus (b). GAPDH gene was amplified by PCR using cDNA derived from total RNA of 50 glomeruli (c). (a) Bar = 50 μm. (c) Lane 1: molecular weight marker, lane 2: cDNA sample derived from 50 glomeruli, lane 3: negative control (no template).

Fig. 1 (a) Sieving method



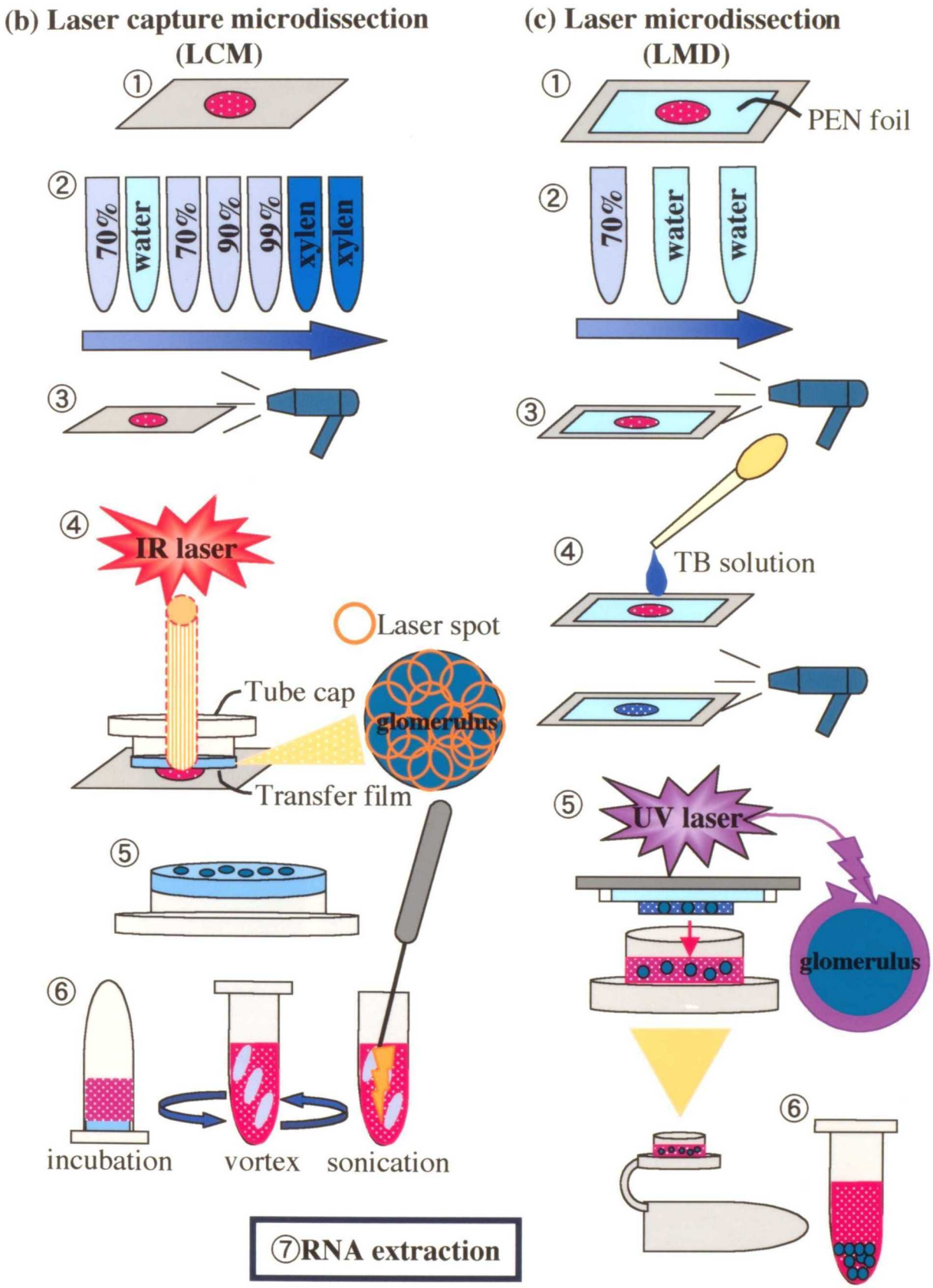

(b) Laser capture microdissection (LCM)
(c) Laser microdissection (LMD)
①
②
70%
water
70%
90%
99%
xylen
xylen
③
④
IR laser
Laser spot
Tube cap
glomerulus
Transfer film
⑤
⑥
incubation
vortex
sonication
⑦RNA extraction
PEN foil
70%
water
water
TB solution
UV laser
glomerulus

Fig. 2

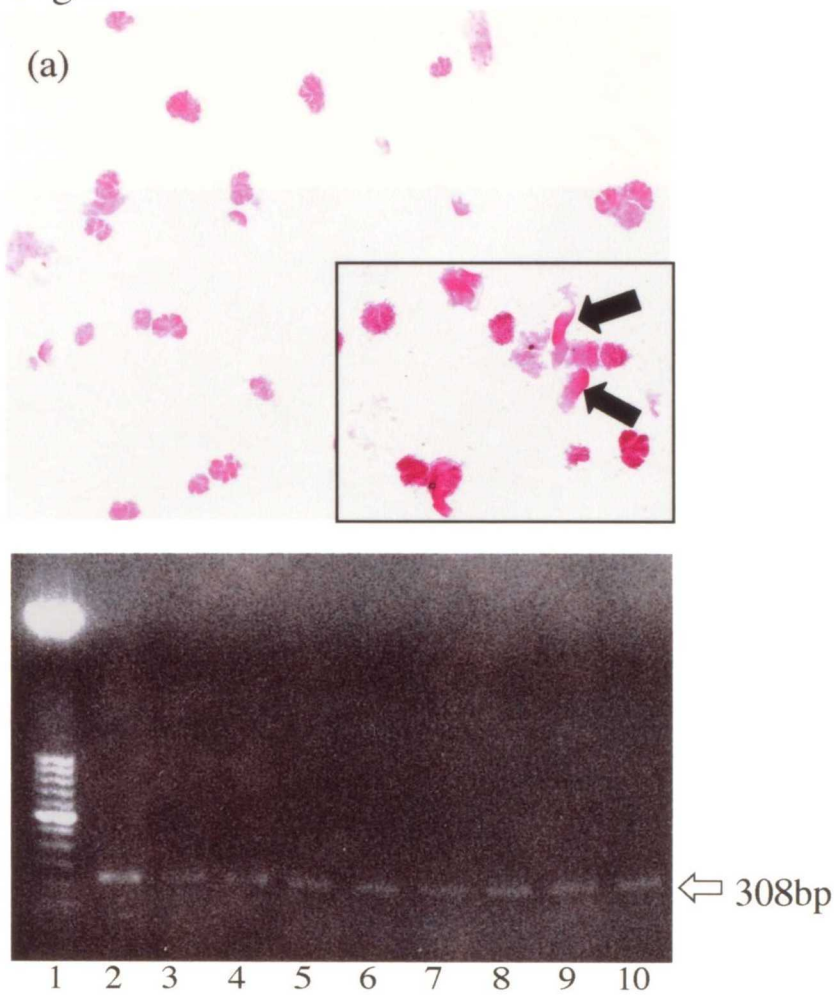


Fig.3

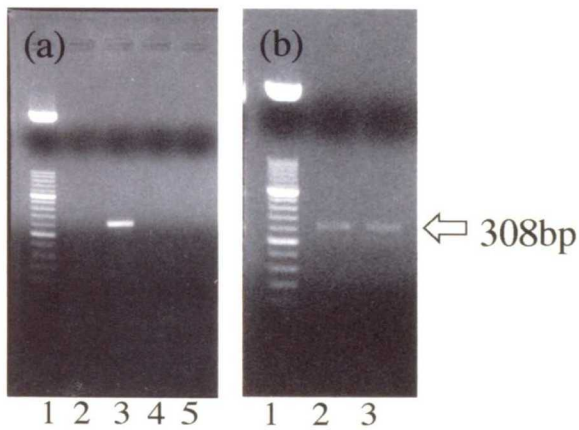
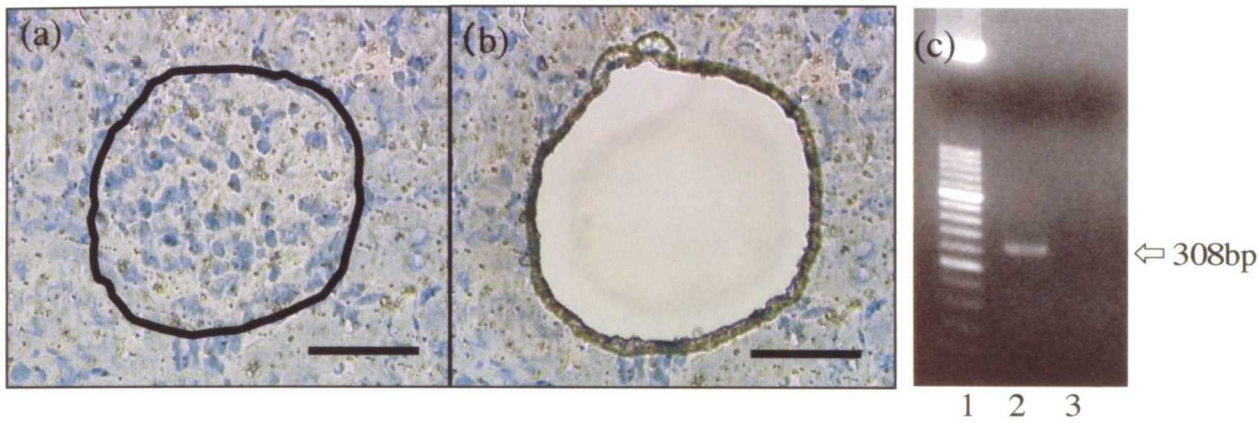


Fig.4



Chapter Ⅱ

Detection of gene expression of VEGF and flk-1 in the renal glomeruli of the normal rat kidney using the laser microdissection system

Introduction

Vascular endothelial growth factor (VEGF) is a growth factor that has significant roles in angiogenesis, tumor growth and development (Ferrara, 1999). It is composed of a group of at least five different molecular species in humans, designated as follows: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Ferrara, 1999). In rats, VEGF is also composed of a group of three isoforms, designated $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$, which are equivalent to $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ in humans, respectively (Ng, *et al.* 2000). There are two specific high-affinity VEGF receptors, flt-1 and KDR/flk-1, which are located on vascular endothelial cells, induce endothelial cell proliferation and increase vascular permeability to macromolecules (Ferrara, 1999). In the renal glomeruli, VEGF is produced by the podocytes in normal and diseased kidneys, activated mesangial cells, infiltrating leukocytes in diseased kidney (Gruden, *et al.* 1997., Brown, *et al.* 1992., Iijima, *et al.* 1993., Noguchi, *et al.* 1998., Masuda, *et al.* 2001.) and endothelial cells *in vitro* (Uchida, *et al.* 1994.). The receptor flk-1 is expressed on normal, damaged and regenerating glomerular endothelial cells (Masuda, *et al.* 2001.) as well as mesangial cells (Thomas, *et al.* 2000.).

The author suspected that VEGF might contribute to the reconstruction of damaged glomeruli by stimulating glomerular cells including glomerular endothelial cells as well as mesangial cells and some reports supported this suspecting. Therefore, the analysis of the gene expression of VEGF and its receptors in the glomeruli is important for understanding the mechanisms of glomerular diseases and for establishing the new therapeutic strategies. With the *in situ* hybridization technique, specific gene expression can be

localized in tissues with high resolution, but the quantitative evaluation of the gene expression is difficult. To perform strict quantitative analysis of gene expression in the glomeruli, the combination of the LMD technique, which was shown the best method to isolate renal glomeruli in Chapter I , and quantitative reverse transcription-polymerase chain reaction (RT-PCR) should be very useful for analyzing gene expression in the glomeruli.

The purpose of this study in this chapter was to detect the local mRNA expression of VEGF and flk-1 by RT-PCR in the glomeruli from normal rat kidney frozen sections using the LMD system in order to show the usefulness of this system for further applications to renal pathological research.

Materials and Methods

Two normal 8-week-old male Wistar rats (Charles River Japan, Inc., Yokohama, Japan) were sacrificed by bleeding under anesthesia and their kidneys were collected. A part of the renal cortex was quickly frozen in liquid nitrogen and stored at -80℃ until use. Frozen sections (5 μm) of the kidneys were made and mounted on glass slides covered with PEN foil (2.5 μm-thick) (Leica Microsystems, Wetzlar, Germany) for the microdissection system used in this study. They were fixed with 70% ethanol at -20℃ for 10 seconds, gently washed twice with diethylpyrocarbonate (DEPC)-treated water, and thoroughly air-dried. After that, the sections were stained with 0.05% toluidine blue (TB) solution, pH 4.1, (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 seconds, and then the TB solution was rinsed out with DEPC-treated water, and the sections were air-dried. Then the renal glomeruli were dissected from the frozen sections with the LMD system using a 337nm- nitrogen ultraviolet (UV) laser (Leica Laser Microdissection System, Leica Microsystems, Wetzlar, Germany) (Fig.1a and b). The glomeruli were dissected from a section and dropped immediately into a microcentrifuge tube cap filled with 30 μl of TRIzol reagent (Life Technologies, Rockville, MD, U.S.A.). Two hundred glomeruli were collected into a 0.5ml tube, and then the total RNA was extracted with TRIzol reagent according to the manufacturer's instructions, using Ethachinmate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for general PCR analysis. First-strand cDNA was made from total RNA using the SuperScript First-strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) with oligo $(dT)_{12\text{-}18}$ primers. PCR was

performed with a TaKaRa PCR Thermal Cycler MP (TaKaRa Shuzo, Kyoto, Japan) with the following oligonucleotide primers: for VEGF (sense: 5'-CCGAATTCACCAAAGAAAGATAGAACAAAG-3' and antisense: 5'-GGTGAGAGGTCTAGTTCCCGA-3'), for flk-1 (sense: 5'-GCCAATGAAGGGGAACTGAAGAC-3' and antisense: 5'-TCTGACTGCTGGTGATGCTGTC-3') and for GAPDH (sense: 5'-TCCCTCAAGATTGTCAGCAA-3' and antisense: 5'-AGATCCACAACGGATACATT-3') (Levy, *et al* 1995., Wen, *et al.* 1998., Ando, *et al.* 1995.). The PCR reaction was performed with Takara *Taq*™ (TaKaRa Shuzo, Kyoto, Japan) under the following conditions: denature at 94℃ for 5 minutes; and then 35 cycles of denature at 94℃ for 45 seconds; annealing at 50℃ for 30 seconds; extension at 72℃ for 30 seconds; and finally extension at 72℃ for 5 minutes. Four microliters of the PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The signal intensity of each band on the gel was evaluated using NIH-image, and the ratios of the levels of the mRNA for the three isoforms of VEGF relative to that of GAPDH mRNA were calculated.

For real-time PCR analysis, 10, 25, 50, 100 and 200 glomeruli were from another three normal Wistar rats using the LMD system. And then the total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, California, U. S. A.) and DNase digestion was performed with RNase-free DNase set (Qiagen, Valencia, California, U. S. A.). First-strand cDNA was made from total RNA using Sensicript (Qiagen, Valencia, CA, U. S. A.) with oligo $(dT)_{15}$ primer (Promega, Madison, WI, U. S. A.). Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster

City, CA, U. S. A.). The TaqMan probe and primer pairs of VEGF and flk-1 were designed using Primer Express version 1.5 (Applied Biosystems, Foster City, CA, U. S. A.) based on their sequence. The VEGF TaqMan probe was 5'TGAAGTTCATGGACGTCTACCAGCGCA3'. The VEGF forward primer sequence was 5'GAGCAGAAAGCCCATGAAGTG3', and its reverse sequence was 5'GGTCTCAATTGGACGGCAAT3'. The flk-1 TaqMan probe was 5'TCTCCGTAGACCTGAAGCGGCGC3'. The flk-1 forward primer sequence was 5'AATTCGTGCCCTATAAGAGCAAA3', and its reverse primer sequence was 5'ACTGCTGGTGATGCTGTCCA3'. GAPDH TaqMan probe, forward and reverse primers were obtained from TaqMan Rodent GAPDH Control Reagents (Applied Biosystems, Foster City, CA, U. S. A.). The PCR reaction mixture consisted of distilled water, TaqMan probes and primers of GAPDH and VEGF or flk-1, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, U. S. A.) and the cDNA from the dissected glomeruli. PCR conditions were as follows: two minutes at 50℃ for uracil-N-glycosylase incubation, 10 minutes at 95℃ for the activation of AmpliTaq Gold DNA polymerase, and then 40 cycles of 95℃ for 15 seconds and 60℃ for one minutes. The amplification plots of VEGF, flk-1 and GAPDH in each sample was analyzed on the point of threshold cycle number and amplification curve to obtain stable quantitative results for the experiments afterwards.

Results

Local expression of rat $VEGF_{120}$, $VEGF_{164}$, $VEGF_{188}$, flk-1 and GAPDH mRNAs was detected in the 200 renal glomeruli from two normal rats, as

shown by RT-PCR products of the expected sizes (Fig.2). Among the three isoforms of rat VEGF, $VEGF_{164}$ and $VEGF_{120}$ had the strongest and weakest mRNA signals, respectively. The ratio of the level of the mRNA of each VEGF isoform to the level of GAPDH mRNA was almost the same in the glomeruli of the two normal rats ($VEGF_{120}$: 0.07 and 0.11, $VEGF_{164}$: 0.61and 0.66 and $VEGF_{188}$: 0.47 and 0.49).

The cDNA of VEGF and GAPDH extracted from 100 and 200 glomeruli began amplifying at 27 to 33 cycles of PCR, respectively, and their plots showed stable and exponential amplification by real-time PCR (Figs. 3d, 3e, 4d and 4e). It was also possible to detect exponential amplification of their cDNA derived from 10, 25 and 50 glomeruli and they began amplifying at 31 to 35 cycles. However, some samples derived from 10, 25 and 50 glomeruli showed unstable amplification, which meant that one of duplicated samples did not amplify completely, or similarly (Figs. 3a to 3c and 4a to 4c). The cDNA of flk-1 derived from 200 glomeruli was also amplified using the same system. Because cDNA of flk-1 began amplifying at over 35 cycles using the same quantity of cDNA as VEGF and GAPDH, it was impossible to obtain enough data to quantify its expression (data not shown). However, it was possible to detect exponential amplification, which could perform enough quantitative analysis for flk-1 using twice as much cDNA as it was used for VEGF and GAPDH (Fig. 5).

Discussion

The LMD technique is very useful for mRNA analysis in the specific cells or tissues. With the LMD system used in this study, we can precisely trace the

outlines of cells or functional units in the tissues and cut them using the UV laser with little tissue damage. We collected the dissected tissues into microcentrifuge tube caps filled with RNA extraction reagent immediately after each cutting, and such system reduced the loss and denatures of biological materials (Kolble, et al. 2000.). Modification of the techniques for RNA extraction and reverse transcription after microdissection may increase the sensitivity.

The expression of the mRNAs of rat VEGF isoforms $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ were detected in the normal renal glomeruli, and that of $VEGF_{164}$ was the most abundant among the isoforms (Merril, et al. 1995.). $VEGF_{164}$ was also the most abundant isoform in mouse glomeruli, whereas $VEGF_{188}$ was the least abundant (Kretzler, et al. 1998.). Moreover, the relative amounts of the mRNA of the VEGF isoforms vary from organ to organ or tissue to tissue in developing and mature mice (Ng, et al. 2000). In the adult mouse kidney, the percentages of the mRNAs of $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ were reported to be 20%, 63% and 17%, respectively (Ng, et al. 2000). Each VEGF isoform has unique characteristics or capacities of receptor binding, heparin binding, localization and activity (Ferrara, 1999). Therefore, it is important to evaluate their different roles in glomerular diseases. We did not determine the ratios of the mRNA expression of the isoforms because the primer pair for real-time PCR was designed to detect all of VEGF isoforms; however, the mRNA of $VEGF_{164}$ was most abundant in RT-PCR. The ratio of each VEGF isoform mRNA to GAPDH mRNA was almost the same in the renal glomeruli of two normal rats in this study. This indicates the reproducibility of the analysis of mRNA expression in the renal

glomeruli using the LMD system.

For the real-time PCR, we used 200 glomeruli and performed the RNA extraction and reverse transcription reaction using different reagents from ones that used for standard PCR as indicated by Boylan *et al* (2001). The amplification plots of cDNA of VEGF and GAPDH revealed that cDNA derived from more than 100 glomeruli will be sufficient and at least 10 glomeruli might be needed for quantitative analysis of these genes. As to flk-1, the author could obtain sufficient results for quantification of mRNA using twice as much cDNA as it was used for VEGF and GAPDH in this study. It might be due to the construction of primers and TaqMan probe used in this study or little expression of flk-1 mRNA in the normal glomeruli. To quantify mRNA expression of several factors in the glomeruli, using enough cDNA samples or performing multiplex real-time PCR would be best.

Abstract

The recent development of the laser microdissection (LMD) technique enables one to target particular tissues or cells for gene or protein analyses. In the renal glomeruli, VEGF is produced by the podocytes in normal and diseased kidneys, activated mesangial cells, infiltrating leukocytes *in vivo* and endothelial cells *in vitro*. The receptor flk-1 is expressed on normal, damaged and regenerating glomerular endothelial cells as well as mesangial cells. The author suspected that VEGF might contribute the reconstruction of damaged glomeruli to stimulate glomerular cells such as endothelial cells as well as mesangial cells. Therefore, it is important to analyze the gene expression of VEGF and its receptors in the glomeruli for understanding the mechanisms of glomerular diseases and for establishing the new therapeutic strategies. The purpose of this study was to detect local mRNA expression of vascular endothelial growth factor (VEGF) and its receptor, flk-1, in the glomeruli of normal rat kidneys using the LMD system in order to show the usefulness of this system for further applications to renal pathological research. The frozen sections of the kidney of 8-week-old male Wistar rats were made. The glomeruli were dissected from the frozen sections with the LMD system, and total RNA was extracted from 200 glomeruli in each kidney. RT-PCR revealed the local mRNA expression of three isoforms of VEGF, flk-1 and GAPDH in the glomeruli. Moreover, the real-time PCR was performed to evaluate the experimental condition for quantification of VEGF and flk-1 mRNA expression using this system, and the results showed that at least 10 glomeruli might be needed for quantifying local VEGF mRNA expression. And it was possible to perform quantitative evaluation of flk-1 mRNA using

twice as much cDNA derived from 200 glomeruli as it was used for VEGF and GAPDH with this system. These results demonstrate the reproducibility of the analysis of mRNA expression in the renal glomeruli using the LMD system and also suggest that the application of the LMD technique will provide information to further our understanding of the mechanisms of kidney diseases.

Figure Legends

Fig.1: Collection of the renal glomeruli by Laser microdissection (LMD) system. The renal glomerulus in the frozen section stained with TB solution was exactly traced on the monitor with the computer mouse as indicated by the line (a). Then it was dissected with the ultraviolet laser (b), and the dissected glomeruli were directly dropped into microcentrifuge tube cap filled with a reagent for RNA extraction immediately after each dissection in the LMD system used in this study. Bars 50 μ m (a, b).

Fig.2: The mRNA expression of rat VEGF120, VEGF164, VEGF188, flk-1 and GAPDH in the two normal rat glomeruli collected by the LMD system. Most intense signal among VEGF isoforms was of $VEGF_{164}$.

Fig. 3: The amplification plots of GAPDH.
The exponential amplification of GAPDH cDNA derived from 10 (a), 25 (b), 50 (c), 100 (d) and 200 glomeruli (e) by real-time PCR. The amplification curves expressed by three different colors are of duplicated samples from three normal rats.

Fig. 4: The amplification plots of VEGF.
The exponential amplification of VEGF cDNA derived from 10 (a), 25 (b), 50 (c), 100 (d) and 200 glomeruli (e) by real-time PCR. The amplification curves expressed by three different colors are of duplicated samples from three

normal rats.

Fig. 5 The amplification plots of flk-1.

The exponential amplification of flk-1 cDNA derived from 200 glmeruli by real-time PCR. These plots were obtained using twice as much cDNA as it was used for VEGF and GAPDH. The amplification curves expressed by three different colors are of duplicated samples from three normal rats.

Fig. 1

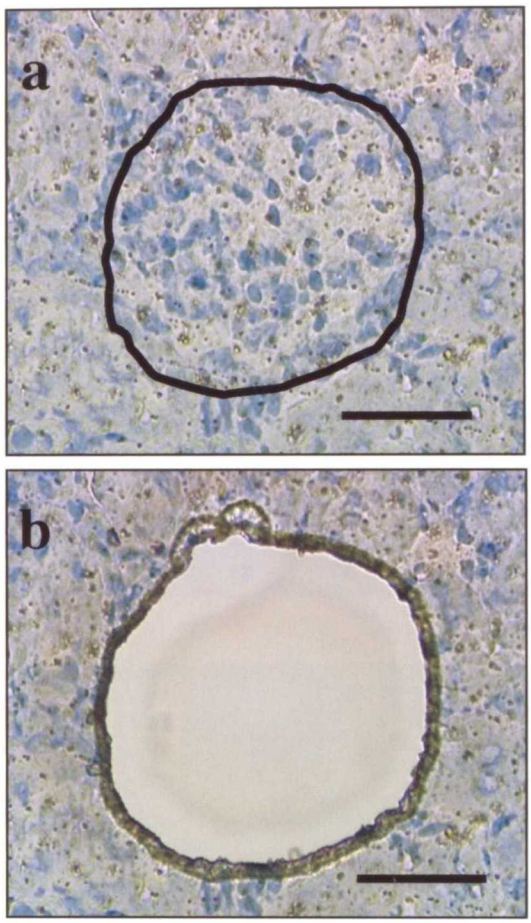

a
b

Fig. 2

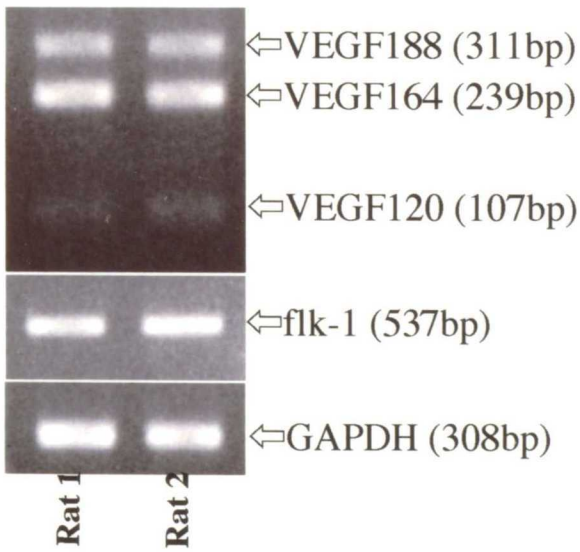
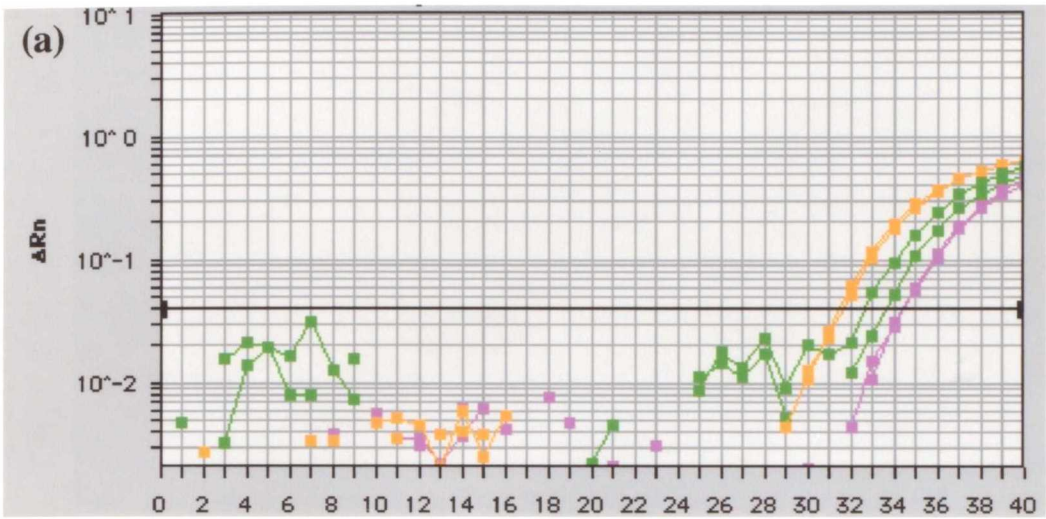

⇦VEGF188 (311bp)
⇦VEGF164 (239bp)
⇦VEGF120 (107bp)
⇦flk-1 (537bp)
⇦GAPDH (308bp)
Rat 1
Rat 2

Fig. 3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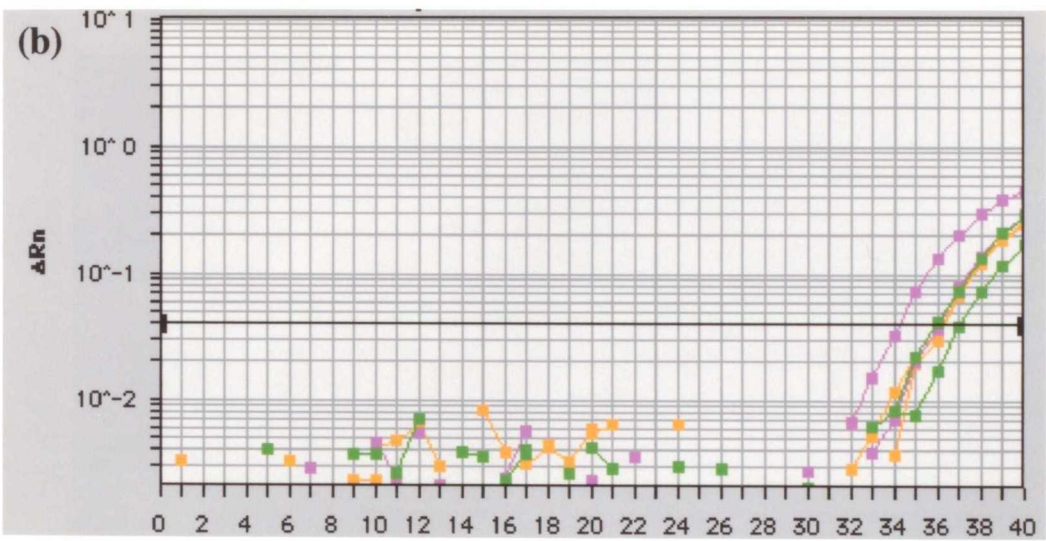
10^1
10^0
10^-1
10^-2
ΔRn
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40


(b)
10^1
10^0
10^-1
10^-2
ΔRn
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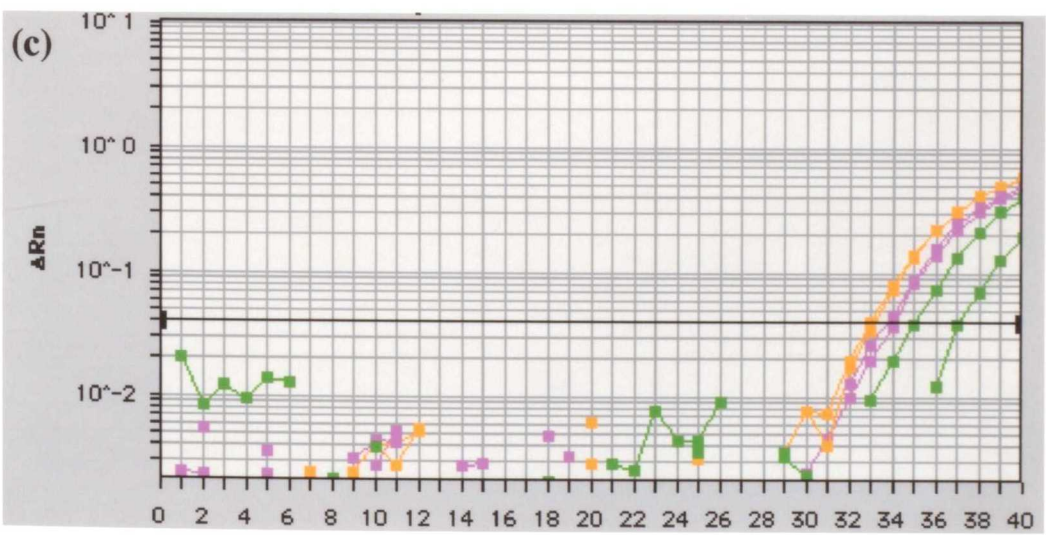
(c)
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10^0
10^-1
10^-2
ΔRn
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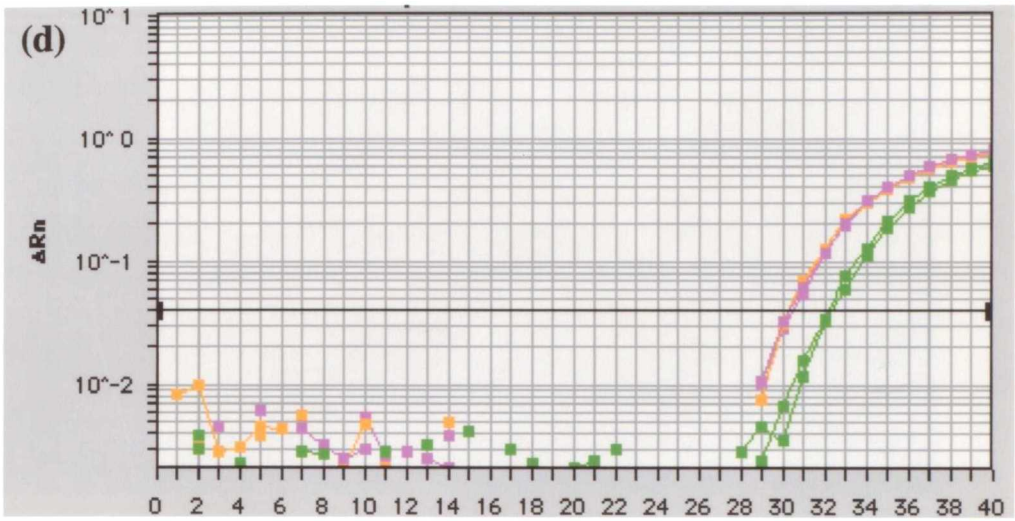
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10^-1
10^-2
ΔRn
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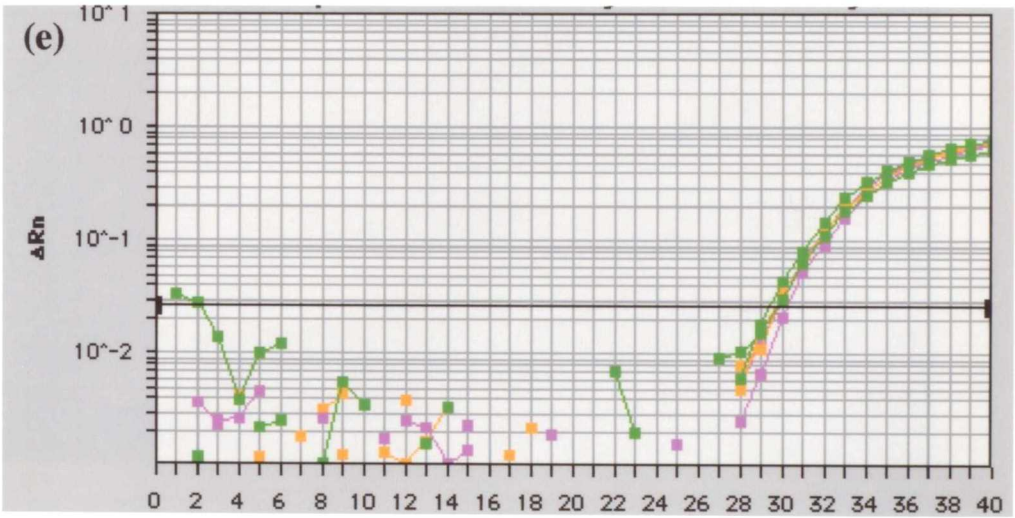
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10^-1
10^-2
ΔRn
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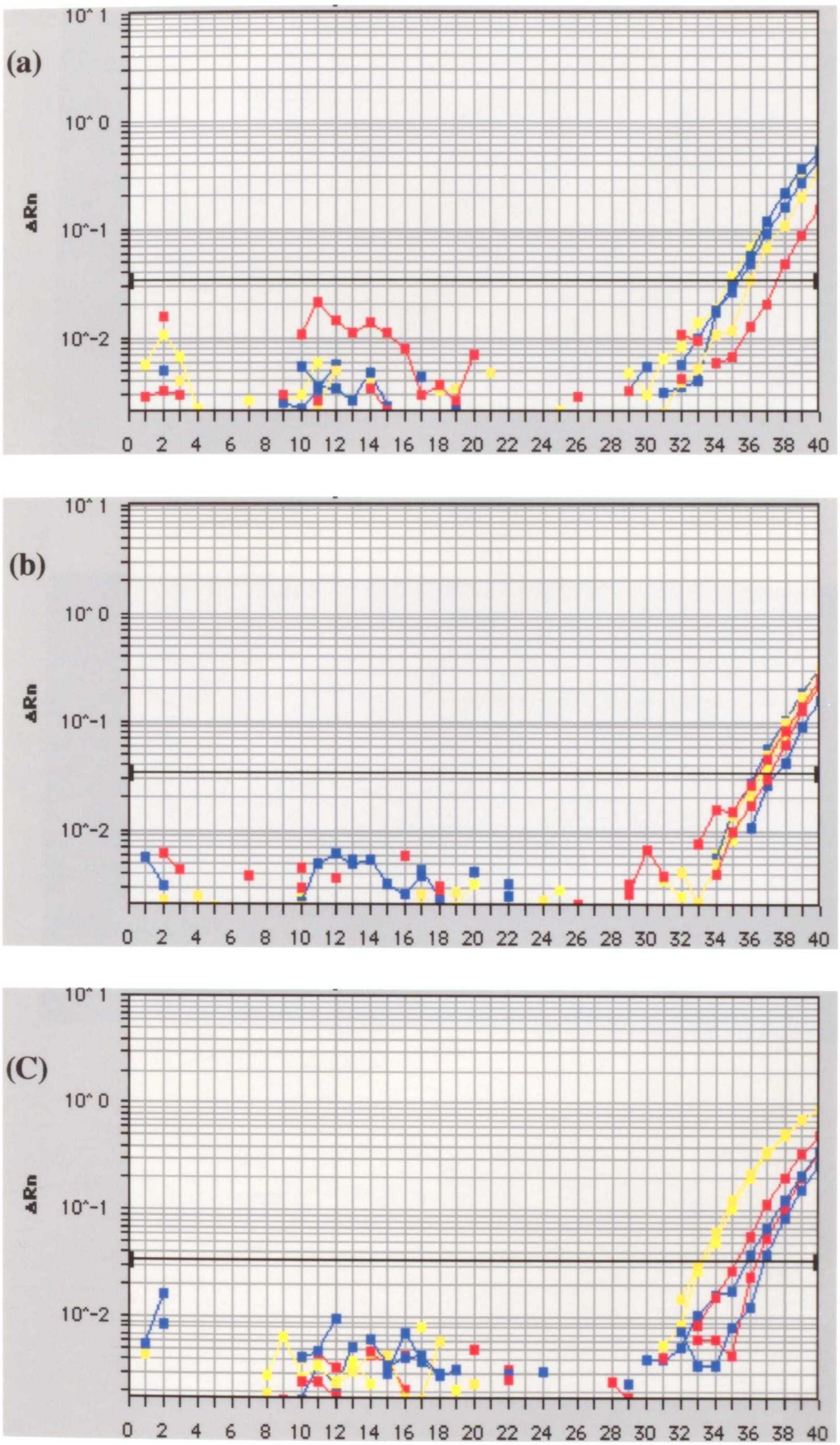
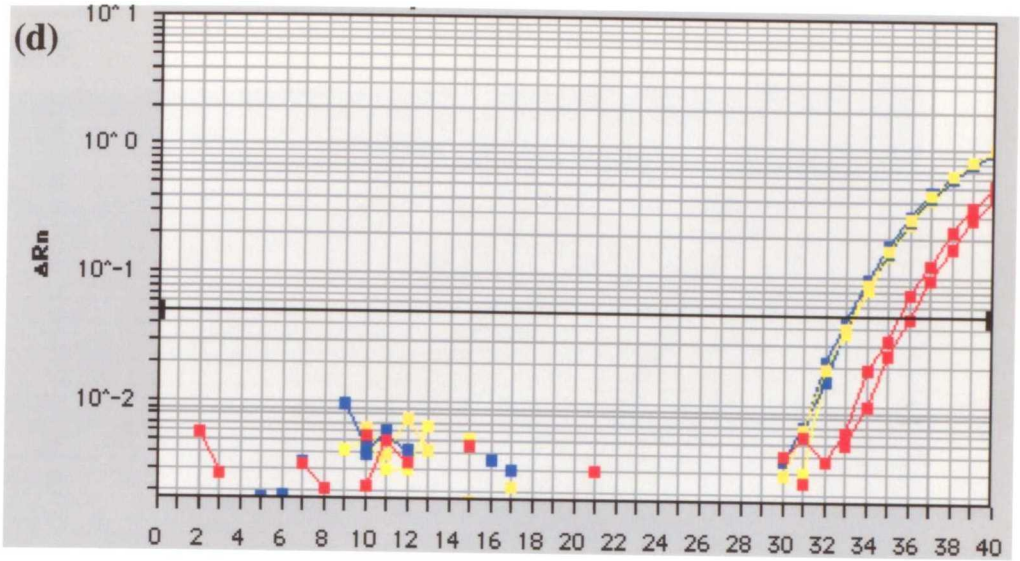
Fig. 4


(a)
(b)
(C)
ΔRn
10^1
10^0
10^-1
10^-2
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40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10^ 0
10^-1
10^-2
ΔRn
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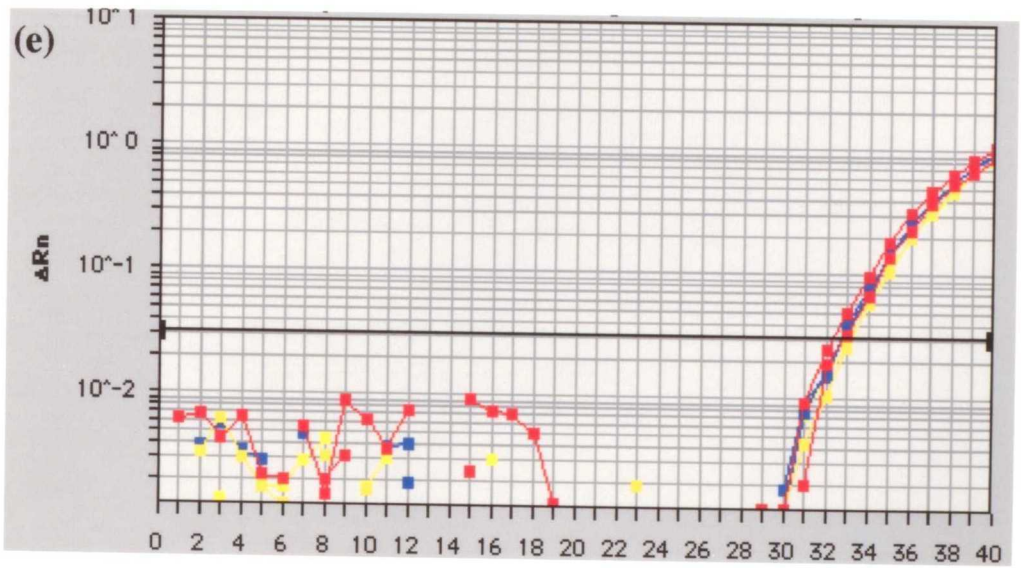
(e)
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10^-1
10^-2
ΔRn
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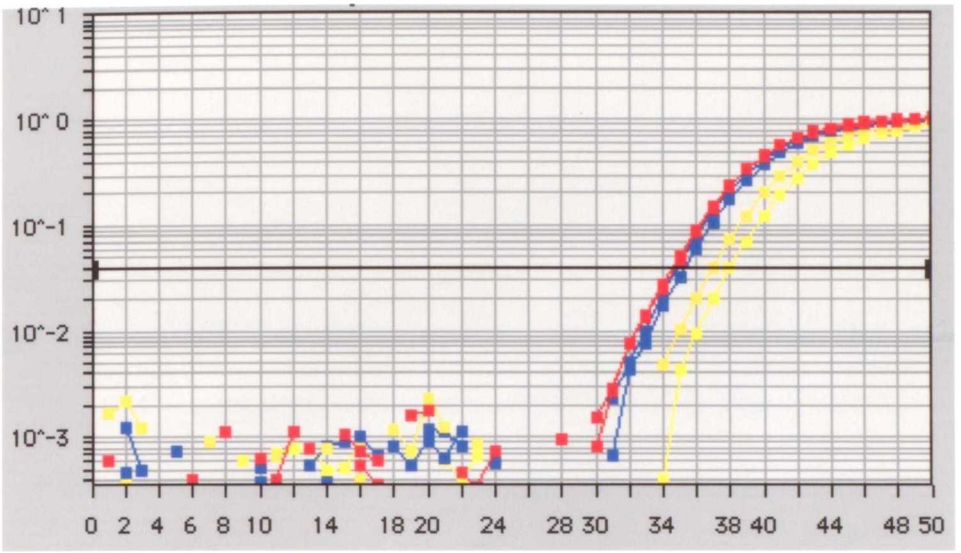
Fig. 5



Chapter Ⅲ

The role of VEGF and flk-1 on the recovery of the glomerular structure in anti-Thy-1 nephritis

Introduction

One of the angiogenic growth factors, vascular endothelial growth factor (VEGF) has significant roles in angiogenesis, tumor growth and development (Ferrara, 1999.). There are two specific high-affinity VEGF-receptors designated as flt-1 and KDR/flk-1, which are located in the vascular endothelial cells, induce endothelial cell proliferation and increase vascular permeability to macromolecules (Ferrara, 1999.).

In the renal glomeruli, VEGF is produced by the podocytes in normal and diseased kidneys, activated mesangial cells, infiltrating leukocytes (Brown, *et al.* 1992., Gruden, *et al.* 1997., Iijima, *et al.* 1993., Noguchi, *et al.* 1998., Shulman, *et al.* 1996., Thomas, *et al.* 2000.) and endothelial cells in vitro (Uchida, *et al.* 1994.). The receptor flk-1 is expressed in normal, damaged and regenerating glomerular endothelial cells (Masuda, *et al.* 2001.) as well as mesangial cells (Thomas, *et al.* 2000.).

The anti-Thy-1 nephritis is induced by a single intravenous injection of either anti-thymocyte antibody or complement fixing monoclonal or polyclonal antibody to the Thy-1 antigen (Yamamoto, *et al.* 1987., Bagchus, *et al* 1986., Johnson, *et al.* 1990). Thy-1 antigen is expressed in a variety of cell types in addition to rat thymocytes (Barcley, *et al.* 1981.) and can also be detected on the surface of mesangial cells in rats (Yamamoto, *et al.* 1986.). Injection of anti-Thy-1 antibody into rats results in rapid, complement-dependent loss of mesangial cells with disruption of the mesangial matrix ("mesangiolysis") (Yamamoto, *et al.* 1987., Bagchus, *et al* 1986., Johnson, *et al.* 1990). The early mesangiolysis is followed by massive mesangial cell proliferation, peaks from day 3 to day 7 and quickly subsides thereafter. Mesangial cell proliferation is associated with

phenotypic changes of these cells, as shown by the *de novo* expression of α-smooth muscle actin in mesangial cells and the *de novo* appearance of interstitial type I collagen in the mesangial matrix (Johnson, *et al.* 1991.). Besides interstitial type I collagen, marked glomerular accumulation of various other extracellular matrix components occurs in parallel with the mesangial proliferative phase (Floege, *et al.* 1991.). The disease is usually reversible, unless anti-Thy-1 antibody is repeatedly injected, in which case focal or global glomerulosclerosis develops (Stahl, *et al* 1990., Iida, *et al.* 1993.). In human mesangioproliferative diseases, mesangiolysis has been recognized (Morita, *et al.* 1983.) and the occurrence of anti-mesangial cell autoantibodies has been documented in IgA nephropathy (O'Donoghue, *et al.* 1991.). Thus, the mechanisms involved in the pathogenesis of mesangioproliferative anti-Thy-1 nephritis in the rat have counterparts and are likely to be of pathogenic importance in human glomerular disease.

Recently, many studies have been performed to examine the mechanisms of mesangial proliferation and matrix accumulation in anti-Thy-1 nephritis. However, in these years, a "resolution" phase of anti-Thy-1 model has been studied to understand the mechanisms of spontaneous glomerular capillary repair after the disruption of glomerular structure. The experimental studies showed that the glomerular endothelial cell was an active participant in the capillary repair and restoration of glomerular architecture. It was also indicated that VEGF played an important role in endothelial cell proliferation and capillary repair in damaged glomeruli in anti-Thy-1 nephritis. (Masuda, *et al.* 2001., Iruela-Arispe, *et al.* 1995., Ostendorf, *et al.* 1999.). The author of this thesis also suspected that VEGF might greatly contribute to the reconstruction or repair of injured

glomeruli. However, suitable method has not been performed as regards the quantitative analysis of VEGF and flk-1 mRNA expression in the glomeruli of anti-Thy-1 nephritis in the previous studies.

In chapters 1 and 2, the author evaluated the method for glomerular isolation and quantitative analysis of VEGF and flk-1 mRNA expression in the glomeruli, and showed that the combination of LMD system and real-time PCR enabled us to quantify their mRNA expression in the glomeruli more strictly. Therefore, in this chapter, to clarify the role of VEGF and its receptor in the glomeruli of anti-Thy-1 nephritis, the detailed quantitative evaluation of gene expression was performed using these techniques. Moreover, we examined their localization in the renal tissue using immunohistochemical analysis and *in situ* hybridization analysis.

Materials and Methods

Anti-Thy-1 glomerulonephritis model of rats

For the induction of anti-Thy-1 nephritis (anti-Thy-1 nephritis group), 8-week-old male Wistar rats (Charles River Japan, Inc., Yokohama, Japan) were injected intravenously with 0.5 ml / 100g body weight of polyclonal anti-Thy-1 antibody produced in rabbits immunized with rat thymocytes as described previously (Ishizaki, *et al.* 1986.). As the control group, rats were injected with normal rabbit serum in stead of anti-Thy-1 antibody. Rats were sacrificed by bleeding under ether anesthesia on days 3, 7, 14, 28 and 56 after the administration of anti-Thy-1 antibody or normal rabbit serum (n = 4 to 6 per time point) and their kidneys were collected.

Histopathological and immunohistochemical examinations.

After removal of the kidney, renal tissues were fixed in 10% formalin neutral--buffered solution, Mildform (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin. The sections (3 μ m) were stained with hematoxylin and eosin (H-E) and periodic acid-Schiff (PAS) for light microscopic examination.

For immunohistochemistry, paraffin sections were used, and the specimens were stained by the streptoavidin-biotin-peroxidase complex technique using Histofine kit (Nichirei Corp., Tokyo, Japan). The following antibodies were used as primary ones: 1) monoclonal mouse anti-human α-smooth muscle actin (α-SMA) antibody (1A4; DAKO, Glostrup, Denmark), which is a marker for activated mesangial cells; 2) monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody (PC10, DAKO), which is a marker for proliferating

cells; 3) monoclonal mouse anti-rat monocytes-macrophages antibody (ED1; Serotec, Sapporo, Japan); 4) polyclonal rabbit anti-VEGF antibody (Takara, Ohtsu, Japan), which can detect VEGF-producing cells; 5) Monoclonal mouse anti-flk-1 antibody (A-3; Santa Cruz Biotechnology, Santa Cruz, CA), which can detect cells that express VEGF receptor; 6) monoclonal mouse anti-desmin antibody (ZC18; Nichirei Corp. Tokyo, Japan), which can detect injured podocytes (Yaoita, *et al.* 1990.). For the staining of ED-1, α-SMA and desmin, 4% paraformaldehyde (PFA)-fixed paraffin sections were used. For the staining of α-SMA and PCNA, tissue sections were treated by microwave for 10 min at 90℃ in 10mM citrate buffer, pH 6.0 after deparaffinization. Also the tissue sections were pretreated with 0.1% trypsin for 30 min at 37℃ and pepsin solution (Nichirei Corp., Tokyo, Japan) for 20 min at 37℃ for ED-1 and anti-flk-1 antibody, respectively. The immunoreaction was visualized by a diaminobendine-hydrogen peroxidase solution, and the sections were counterstained with hematoxylin. As regards flk-1, the immunoreaction in some samples was strengthened by DAB ENHANCING SOLUTION (Vector laboratories, Burlingame, CA, USA). As regards PCNA and ED-1, the number of the positive cells in 100 glomeruli were counted and calculated the mean number of positive cells in a glomerulus.

Laser microdissection, RNA extraction and reverse transcription reaction.

A part of the renal cortex of each rat was quickly frozen in liquid nitrogen and stored at –80℃ until use. Frozen sections (8 μ m) of the kidneys were made and mounted on glass slides covered with PEN foil (2.5 μ m thick; Leica Microsystems, Wetzlar, Germany) for the microdissection system used in this study. They were fixed with 70% ethanol for 10sec, gently washed twice with diethylpyrocarbonate (DEPC)-treated water, and thoroughly air-dried. After that, the sections were stained with 0.05% toluidine blue (TB) solution, pH 4.1, (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10sec, and then the TB solution was rinsed with DEPC-treated water, and the sections were air dried. Then renal glomeruli were dissected from the frozen sections with the LMD system using a 337-nm nitrogen ultraviolet (UV) laser (Leica Microsystems, Wetzlar, Germany). The glomeruli dissected from a section dropped immediately into a tube cup filled with 50 μ l RNA extraction buffer. Two-hundreds glomeruli, which is the enough number to quantify mRNA expression in the glomeruli (Inoue, *et al.* 2003), were collected into a 0.5-ml tube. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and DNase digestion was performed with RNase-free DNase set (Qiagen). First strand cDNA was made from total RNA using Sensiscript (Qiagen) with oligo $(dT)_{15}$ primer (Promega, Madison, Wis., USA).

Real-time PCR

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). The TaqMan probe and primer pair of VEGF was designed to detect VEGF164, which is most

abundant among the isoforms (Merril, *et al.*1995.), and the mRNA of this isoform was most abundant in the normal rat glomeruli (Inoue, *et al.* 2003). The probe and primer pair for flk-1 was designed using Primer Express version 1.5 (Applied Biosystems) based on their sequence. These sequences of probes and primers were listed in Table 1. GAPDH TaqMan probe, forward and reverse primers were obtained from TaqMan Rodent GAPDH Control Regents (Applied Biosystems). The PCR reaction mixture consisted of distilled water (D. W.), TaqMan probes and primers of GAPDH, VEGF or flk-1, TaqMan Universal PCR Master Mix (Applied Biosystems) and the cDNA from the dissected glomeruli. PCR conditions were as follows: 2 min at 50℃ for uracil-N-glycosylase incubation, 10 min at 95℃ for the activation of AmpliTaq Gold DNA polymerase, and then 50 cycles of 95℃ for 15 sec and 60℃ for 1 min. The amplification plots of VEGF, flk-1 and GAPDH in each sample was analyzed on the point of threshold cycle number, and the relative quantity of GAPDH and VEGF or flk-1 was analyzed using the Comparative Ct Method described in User Bulletin＃2: ABI PRISM 7700 Sequence Detection System.

. Cloning, sequencing and preparation of probes for *in situ* hybridization

Total RNA was isolated from the cortex of the normal rat kidney using TRIzol regent (Life Technologies, Rockville, Md., USA). First strand cDNA was synthesized by reverse transcription (RT) of 3 μ g total RNA using the Superscript First-strand Synthesis System (Invitrogen Life Technologies, Carlsbad, Calif., USA). Using the primers for VEGF and flk-1 with Takara *Taq* (Takara Shuzo, Ohtsu, Japan), the corresponding cDNAs were amplified from the RT product by polymerase chain reaction in a Takara PCR Thermal Cycler

MP (Takara Shuzo). The oligonucleotide primers sequences were as follows; VEGF primers (sense: 5'-CCGAATTCACCAAAGAAAGATAGAACAAAG-3' and antisense: 5'-GGTGAGAGGTCTAGTTCCCGA-3'), and flk-1 primers (sense: 5'-GCCAATGAAGGGGAACTGAAGAC-3', and antisense: 5'-TCTGACTGCTGGTGATGCTGTC-3')(Levy, *et al.* 1995., Wen, *et al.* 1998.). The PCR products were then applied for electrophoresis in a 2% agarose gel and stained with ethidium bromide to confirm their specific size bands. After the electrophoresis, the PCR products were purified and subcloned into the TA cloning site of plasmid pGEM-T easy (Promega, Madison, Wisc., USA). The cDNA sequences of the inserts for VEGF164 (239bp) and flk-1 (537bp) were confirmed by sequencing. Briefly, the inserts were treated with exonuclease I (Amersham Biosciences Corp. Piscataway, NJ, USA) and shrimp alkaline phosphatase (Amersham Biosciences Corp.) at 37℃ for 25 min and then placed at 80℃ for 15 min to inactivate the enzymes. The reaction products were directly sequenced for both forward and reverse strands with the amplification primers for T7 and SP6, employing the AmpliTaq Dye Terminator Cycle Sequencing FS kit (PerkinElmer Life & Analytical Sciences, Inc. Boston, MA, USA) on a 373A DNA sequencer (Applied Biosystems, Foster, CA, USA). After sequencing, the inserts for VEGF164 and flk-1 were excised from the vectors by using the restriction enzymes Nco I and Nde I (Promega), and then digoxigenin-labeled sense and antisense RNA probes (riboprobes) were prepared in vitro with T7 or SP6 polymerase, respectively using MAXIscript™ in vitro transcription kit (Ambion Austin, Texas, USA) in the presence of digoxigenin (DIG)-conjugated UTP (Roche, Mannheim, Germany).

In situ hybridization

A part of the renal cortex was fixed in 4% paraformaldehyde (PFA) at 4℃ overnight, and paraffin-embedded kidney specimens from normal and anti-Thy-1-treated rats were cut into 4-μm sections and mounted silane-coated glass slides. After deparaffinization and dehydration, the sections were incubated with 100μg/ml proteinase K in PBS buffer at 37℃ for 20 min, and were washed in PBS containing 2mg/ml glycine. Then they were acetylated with acetylation buffer for 15 min, and were prehybridized in a mixture composed of 50% deionized formamide, 2×SSC at 60℃ for 30 min. After prehybridization, the sections were hybridized with the digoxigenin (DIG)-labeled sense or antisense probes for VEGF164 or flk-1 in the hybridization buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 16-24hr at 60℃. After hybridization, the sections were washed with three changes of 0.2×SSC buffer for 20 min at 60℃. Then they were rinsed briefly in TE buffer and for 30 min. at room temperature and rinsed again in TE buffer. Positive signals were detected using DIG Nucleic Acid Detection Kit (Roche). Briefly, the sections were incubated with blocking reagent in TE buffer for 30 min at room temperature and rinsed again with TE buffer. And then incubated 1hr at 37℃ with alkalinephosphatase-conjugated sheep anti-digoxigenin antibody at 1:2500 dilution in the blocking buffer. After two 15-min washes in TE buffer and a brief rinse in the detection buffer, the immunoreaction was visualized by incubating the sections with a NBT/BCIP substrate containing 5-bromo-4-chrolo-3-indolyl-phosphate and nitroblue tetrazolium chloride overnight at room temperature. To confirm whether the infiltrating macrophages and activated mesangial cells produce VEGF, the mirror sections of the kidneys were made and used for immunohistochemistry of

ED-1 or α-SMA and *in situ* hybridization of VEGF.

Statistical analysis

Data were expressed as mean $\pm$ SD. Statistical significance was evaluated by the Mann-Whitney U-test or the Student's t-test. A value of $P<0.05$ was taken to denote statistical significance.

Table 1. Probes and primers used for real-time PCR.

Target sequence	Primer/Probe	sequence
$VEGF_{164}$	Forward primer	5'-CATGCGGATCAAACCTCACC-3'
	TaqMan probe	5'-AAGCCAGCACATAGGAGAGATGAGCTTCC-3'
	Reverse primer	5'-CTGAACAAGGCTCACAGTGATTTT-3'
flk-1	Forward primer	5'-AATTCGTGCCCTATAAGAGCAAA-3'
	TaqMan probe	5'-TCTCCGTAGACCTGAAGCGGCGC-3'
	Reverse primer	5'-ACTGCTGGTGATGCTGTCCA-3'

Results

Histopathological examination of anti-Thy-1 nephritis.

In the rats injected with anti-Thy-1 antibody, mesangial lesions including mesangiolysis were observed on day 3(Fig. 1a), and the glomeruli showed microaneurysm (Fig. 1b) and intensive diffuse or segmental cell proliferation on day 7 (Fig. 1c). Proliferative lesions still remained in some glomeruli although some of them were being repaired on day 14 (Fig. 1d). Almost all the glomeruli were repaired by day 56 (Fig. 1e).

Proliferating cells in the glomeruli of anti-Thy-1 nephritis.

The mean number of PCNA-positive cells in a glomerulus gradually increased from day 3, and reached the maximum on day 7, and then reduced and there were no remarkable change after day 28 (Fig. 2). The mean numbers of PCNA positive cells in a glomerulus were 3.025, 6.672, 3.525, 1.840 and 2.110 on day 3, 7, 14, 28 and 56, respectively. There were significant difference between anti-Thy-1 group and control group on days 7, 14 and 28 ($p<0.05$). Compared with control group, the mean number of PCNA positive cells was almost the same on day 56.

The number of infiltrating macrophages in the glomeruli.

Infiltrating macrophages were observed in the glomeruli of both control and anti-Thy-1 nephritis groups. However, they were rarely observed in control groups and the mean number of ED-1 positive cells showed less than 1.00 (Fig. 3). In anti-Thy-1 nephritis group, the number of ED-1 positive cells was maximum on day 3, and then gradually reduced and returned to the control level

by day 28. There were significant differences between anti-Thy-1 nephritis groups and control groups on days 3, 7 and 14.

Immunohistochemistry of desmin in anti-Thy-1 nephritis.

Desmin-positive cells in the glomeruli were observed only in anti-Thy-1 nephritis and they located in proliferating lesions and some podocytes (Fig. 4). In proliferating lesion, desmin were located not only in the center part, but also at the edge of the lesion (Fig. 4).

Immunohistochemistry of VEGF and flk-1 in anti-Thy-1 nephritis

VEGF was localized only in the podocytes throughout experimental periods in control and nephritic kidneys (Fig. 5a to e). There were no VEGF positive cells in the proliferating foci in the mesangium on day 7 (Fig. 5c). Some elongated podocytes in the enlarged glomeruli did not express VEGF (Fig. 5c).

Flk-1 was located in the glomerular endothelial cells in the normal and diseased kidney (Fig. 6a to e). In anti-Thy-1 nephritis, flk-1 was observed in the endothelial cells located in some microaneurysm and the opened capillaries in the glomeruli on day 7 to day56 (Figs. 6b to e). There were no positive cells in the proliferating foci in the mesangium on day 7 and 14 (Figs. 6c and d).

Quantitative analysis of VEGF and flk-1 mRNA expression in the glomeruli.

In anti-Thy-1 nephritis, VEGF mRNA level was significantly lower than that of control groups on day 3, reached the minimum on day 7, and then significantly increased and became to the control level on days 28 and 56 (Fig. 7). Significant differences between nephritic and control rats were observed on days

3, 7 and 14 ($p<0.05$). There were also significant differences between each anti-Thy-1 nephritis group from day 3 to 28 ($p<0.05$).

The level of flk-1 mRNA was lower than that of control group on day 3, significantly reduced and reached to minimum on day 7 and then gradually increased and became to the control level on day 14 (Fig. 8). Thereafter, the level became significantly high on day 28 and decreased to the control level on day 56. Significant difference between nephritic and control rats was observed on days 7 and 28 ($p<0.05$). There were also significant differences between each anti-Thy-1 nephritis groups from day 3 to day 28 ($p<0.05$).

Localization of VEGF mRNA in anti-Thy-1 nephritis by *in situ* hybridization.

VEGF mRNA was detected only in the podocytes in both anti-Thy-1 nephritis groups and control groups throughout the experimental periods (Fig. 9). There was no positive reaction in the proliferative foci consisting of α-SMA positive mesangial cells on day 7 and 14 (Figs. 9b to d). Infiltrating macrophages were observed in the glomeruli of anti-Thy-1 nephritis groups on day 3, however, they did not express VEGF mRNA (Fig. 9e).

Discussion

In this study, the author demonstrated that VEGF and flk-1 did not express in proliferative foci mostly consisting of mesangial cells in anti-Thy-1 nephritis by immunohistochemistry and/or *in situ* hybridization. The levels of VEGF and flk-1 mRNA was minimum in the proliferation phase on day 7 when the number of PCNA positive cells was maximum. Because the quantitative data for mRNA was calculated a relative Ct for VEGF or flk-1 to that for GAPDH, the value may become smaller when the cell number in the glomeruli increased. But we confirmed that proliferating cells, which were mainly mesangial cells, did not express VEGF mRNA by in situ hybridization. Therefore, the quantitative data might indicate that their levels were actually low.

Mesangial proliferation is one of the key features of various glomerular diseases including IgA nephropathy and membranoploliferative glomerulonephritis in human and animals (Churg, *et al.* 1995., Inoue, *et al.* 2001). The anti-Thy-1 model was established and has been used to study the mechanism of the progression of these human diseases. In anti-Thy-1 nephritis, compliment-dependent loss of mesangial cells with disruption of the mesangial matrix (mesangiolysis) is observed after injection of anti-Thy-1 antibody (Yamamoto, *et al.* 1987., Bagchus, *et al.* 1986., Johnson, *et al.* 1991.). After that, mesangial cells proliferate extensively and phenotypic changes are observed (Johnson, *et al.* 1991.). It has been shown that many kinds of cytokines mediate mesangial cell proliferation, including platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), fibroblast growth factor 2 (FGF2) (Floege, *et al,* 1992., Iida, *et al* 1991., Yoshimura, *et al.* 1991. Okuda, *et al.* 1990.). These cytokines are produced by mesangial cells and infiltrating

macrophages or platelets, and induce mesangial cell proliferation. Moreover, because PDGF specific receptors express in mesangial cells, PDGF may mediate mesangial cell proliferation in autocrine manner (Iida, *et al.* 1991.). As regards VEGF, recent studies have indicated that activated mesangial cells expressed VEGF and flk-1 in human and rats (Masuda, *et al.* 2001.,Noguchi, *et al.* 1998., Thomas, et al. 2000.). However, the author demonstrated that VEGF mRNA and protein were expressed only in the podocytes, and flk-1 protein located in the endothelial cells of capillaries with opened lumens in normal and diseased rats in this study. These results suggest that there may be little possibility of autocrine mechanism of VEGF/flk-1 in rat mesangial cells in anti-Thy-1 nephritis model, and VEGF produced by the podocytes might act on the glomerular endothelial cells by paracrine mechanism in normal and glomerulitis.

The role of infiltrating macrophages seems to be important for the progression of glomerulonephritis because they would produce some kinds of cytokines such as PDGF and FGF-2, which would be involved in mesangial proliferation. Peripheral blood mononuclear cells would also produce VEGF (Iijima, et al. 1993.). It was reported that VEGF were immunohistochemically detected in the infiltrating leukocytes in the glomeruli of anti-Thy-1 nephritis (Masuda, *et al.* 2001.). In the present study, the number of ED-1 positive cells was maximum on day 3 and then gradually reduced and returned to the control level by day 28 in anti-Thy-1 nephritis. However, VEGF was not observed in the infiltrating macrophages but only in the podocytes by immunohistochemistry and *in situ* hybridization in both control and anti-Thy-1 nephritis groups. Therefore, the role of infiltrating macrophages as the VEGF producing cells might not be important in anti-Thy-1 nephritis.

There is the glomerular basement membrane (GBM) between flk-1 expressing glomerular endothelial cells and VEGF producing podocytes. To act on its specific receptor, VEGF has to pass through GBM. GBM contains heparan sulfate proteoglycan (HSPG) and $VEGF_{164}$ has the ability to bind heparin-like molecules because it contains the peptide encoded by exon-7 of the VEGF gene (Ferrara, 1999.). Glypican-1 is the prototype member of the glycosylphosphatidilinositol anchored cell surface HSPGs (David, 1993.), and the only member that is expressed in the vascular system (Rosenberg, *et al.* 1997., Mertens, *et al.* 1992.). It was suggested that glypican-1 on the surface of endothelial cells might act to shuttle $VEGF_{164}$ produced by podocytes across the GBM and could mediate $VEGF_{164}$ to contact and activate signaling flk-1 (Gengrinovitch, *et al.* 1999., Kang, *et al.* 2002.). Therefore, $VEGF_{164}$ produced not in mesangial cells, but in the podocytes acts on flk-1 located in the glomerular endothelial cells through extracellular matrix (ECM) including glypican-1.

In puromysin aminonucleoside (PAN) nephrosis of rats, single or repeated intraperitoneal injection of PAN causes pronounced proteinuria that peaks at 1-2 weeks after the injection and declines gradually thereafter. Morphological changes in the glomeruli of PAN nephritis are restricted to podocytes, which show loss, fusion and flattening of their foot processes (Ryan, *et al.* 1975., Andrews, 1977.). In PAN nephritis, the level of VEGF mRNA decreased in association with the induction of proteinuria, and this change of VEGF mRNA was considered to represent the general decline of podocyte functions probably due to PAN-induced damage of podocytes (Fan, *et al.* 2002.). Moreover, in the irreversible model induced by consecutive injections of PAN and mAb 1-22-3,

which is one of the anti-Thy-1 monoclonal antibodies, podocyte injuries caused progressive mesangial alteration suggesting that podocyte function is important for mesangium and glomerular structure (Morioka, *et al.* 2001.). In the early stage of anti-Thy-1 nephritis, mesangial cells are injured directly by injected antibodies followed by mesangiolysis, microaneurysm and glomerular cell proliferation. At the same time, these lesions might injure the podocytes mechanically, because the podocytes are located outside of the GBM. In the present study, some podocytes at the periphery of the proliferating lesions were positive for desmin, which was reported as the marker of injured podocytes (Yaoita, *et al.* 1990.). It was also indicated that the number and density of glomerular endothelial cells have been shown to decrease (Wada, *et al.* 2002.). In this study, the levels of VEGF and flk-1 mRNA expression gradually reduced, became minimum on day 7 and recovered to the level of control rats until day 56 when the glomerular structure was almost recovered. This change of their mRNA expression may reflect the condition and number of podocytes and glomerular endothelial cells.

Recent studies indicate that in anti-Thy-1 nephritis, administration of $VEGF_{165}$ antagonist significantly reduced glomerular endothelial cell proliferation and inhibited glomerular capillary repair, leading to glomerular sclerosis (Ostendorf, *et al.* 1999.). And it is also indicated that $VEGF_{165}$ significantly enhanced endothelial cell proliferation and capillary repair in the progressive model induced by anti- Thy-1 antibody and Habu-snake vemon injection (Masuda, *et al.* 2001.). It is likely that VEGF has the ability to stimulate capillary endothelial cell proliferation and preserve glomerular capillary loops. In the present study, the glomerular structure was almost recovered by day 56

and VEGF and flk-1 were expressed in the podocytes and endothelial cells, respectively. Moreover, the levels of VEGF and flk-1 mRNA were gradually increased and reached to the level of control rats accompanying with the glomerular reconstruction. From the results of this chapter, the author concluded that VEGF produced in the podocytes might play a role on the recovery of glomerular structure in Thy-1 nephritis in paracrine mechanism. The mesangial cells might not be involved in the glomerular restoration through paracrine mechanisms of VEGF/flk-1 in this model.

Abstract

One of the angiogenic growth factors, vascular endothelial growth factor (VEGF) and its specific receptor, flk-1 are strongly involved in endothelial cell proliferation. In the renal glomeruli, VEGF is produced by the podocytes in normal and diseased kidneys, activated mesangial cells and infiltrating leukocytes, and flk-1 is expressed on normal, damaged and regenerating glomerular cells including mesangial cells in human renal diseases and in rat anti-Thy-1 nephritis. Recent studies indicated that the glomerular endothelial cells were involved in the capillary repair and restoration of glomerular architecture, and VEGF played an important role in endothelial cell proliferation and capillary repair in a resolution phase of anti-Thy-1 nephritis. The author also suspected that VEGF might contribute to the reconstruction or repair of injured glomeruli. However, suitable method has not been performed as regards the quantitative analysis of VEGF and flk-1 mRNA expression in the glomeruli of anti-Thy-1 nephritis in the previous studies. In this chapter, the detailed quantitative evaluation was performed using laser microdissection system (LMD) technique and real-time PCR to clarify the role of VEGF and its receptor in the glomeruli of anti-Thy-1 nephritis. Furthermore, the author examined their localization using immunohistchemistry and *in situ* hybridization analysis.

Anti-Thy-1 nephritis was induced in 8-week-old male Wistar rats and their kidneys were collected on days 3, 7, 14, 28 and 56 after the administration of anti-Thy-1 antibody. Histopathological and immunohistochemical examinations for ED-1, α-SMA, PCNA, VEGF and flk-1 were performed. For quantitative analysis of VEGF and flk-1 mRNA in the glomeruli, 200 glomeruli were obtained using LMD system and total RNA was extracted followed by reverse

transcript (RT) reaction to make first strand cDNA , and real-time PCR was performed using primer pairs and TaqMan probes for VEGF164, flk-1 and GAPDH. Moreover, *in situ* hybridization was performed to examine the localization of VEGF164 mRNA using digoxigenin-labeled riboprobes.

In anti-Thy-1 nephritis, mesangial lesions including mesangiolysis were observed on day 3, and the glomeruli on day 7 showed microaneurysm and intensive mesangial cell proliferation, and almost glomeruli had been reconstructed by day 56. VEGF protein and mRNA were located only in the podocytes, and there were no positive cells in proliferating foci in which α-SMA was positive. Flk-1 was located in the glomerular endothelial cells in control and nephritic kidneys, and there were no positive cells in proliferating foci. The level of VEGF and flk-1 mRNA expression were lower than that of control group on day 3, significantly reduced to the minimum on day 7, and then gradually increased. The level of VEGF mRNA returned to the control level by day 28. The level of flk-1 mRNA increased to the control level on day 14 and became significantly high on day 28 and then returned to the control level on day 56.

The number of ED-1 positive cells infiltrating into the glomeruli was maximum on day 3 and then gradually reduced. However, none of these cells showed positive reaction of VEGF by immunohistochemistry and *in situ* hybridization.

These results suggest that there may be little possibility of autocrine mechanism of VEGF/flk-1 in rat mesangial cells *in vivo*. And the mesangial cells might not be involved in endothelial cell proliferation by paracrine mechanisms of VEGF/flk-1. VEGF mRNA level in the glomeruli of nephritic kidneys was gradually increased, which was accompanied with the glomerular reconstruction suggesting that VEGF might play a role on the recovery of glomerular structure

in anti-Thy-1 nephritis in the paracrine mechanism.

Figure Legends

Fig. 1: Histopathological findings of anti-Thy-1 nephritis.

In the rats injected with anti-Thy-1 antibody, mesangial lesions including mesangiolysis were observed on day 3(a). On day 7, the glomeruli showed microaneurysm (b) and intensive diffuse or segmental cell proliferation (c). Proliferative lesions remained in some glomeruli although some of them were repaired on day 14 (d). Almost glomeruli had been repaired by day 56 (e). (f): the glomerulus of control group. Bar=50 μ m.

Fig. 2: The number of PCNA-positive cells in a glomerulus.

The mean number of PCNA-positive cells in a glomerulus gradually increased from day 3, reached the maximum on day 7 and then reduced. Compared with control group, the level was almost the same on day 56. There were significant difference (*) between anti-Thy-1 group and control group on days 7, 14 and 28 ($p<0.05$).

Fig. 3: The number of ED-1 positive cells in a glomerulus.

In anti-Thy-1 nephritis group, the number of ED-1 positive cells was maximum on day 3, and then gradually reduced and returned to the control level by day 28. There were significant differences (*) between anti-Thy-1 nephritis groups and control groups on days 3, 7 and 14.

Fig. 4: Immunohistochemistry of desmin in anti-Thy-1 nephritis.

Desmin-positive cells in the glomeruli were observed only in anti-Thy-1 nephritis and they located in proliferating lesions (＊) and some podocytes expressed it (arrow). In proliferating lesion, desmin were located not only in the center part, but also at the edge of the lesion (arrowhead). Bar=50 μ m.

Fig. 5: Immunohistochemistry of VEGF in anti-Thy-1 nephritis.

VEGF was localized only in the podocytes throughout experimental periods in control and nephritic kidneys (a: control, b: day 3, c: day 7, d: day14, e: day56 in anti-Thy-1 nephritis). There were no positive cells in the proliferating foci in the mesangium on day 7 (c). Some elongated podocytes in the enlarged glomeruli did not express VEGF (c, arrow). Bar=50 μ m.

Fig. 6: Immunohistochemistry of flk-1 in anti-Thy-1 nephritis.

Flk-1 was located in the glomerular endothelial cells in normal and diseased kidney (a: control, b: day 3, c: day 7, d: day14, e: day56 in anti-Thy-1 nephritis). In anti-Thy-1 nephritis, flk-1 was observed in the endothelial cells located in some microaneurysm and the opened capillaries in the glomeruli on day 7 to day56 (b～e). There were no positive cells in the proliferating foci in the mesangium on day 7 and 14 (c and d). Bar=50 μ m.

Fig. 7: Quantitative analysis of VEGF mRNA expression in the glomeruli.

In anti-Thy-1 nephritis, VEGF mRNA level was lower than that of control groups on day 3 and gradually reduced and reached the minimum on day 7.And then the level significantly increased and returned to the control level by day 28. Significant differences (＊) between nephritic and control rats were observed on days 3, 7 and 14 ($p<0.05$). There were also significant differences (※) between each anti-Thy-1 group from day 3 to day 28 ($p<0.05$).

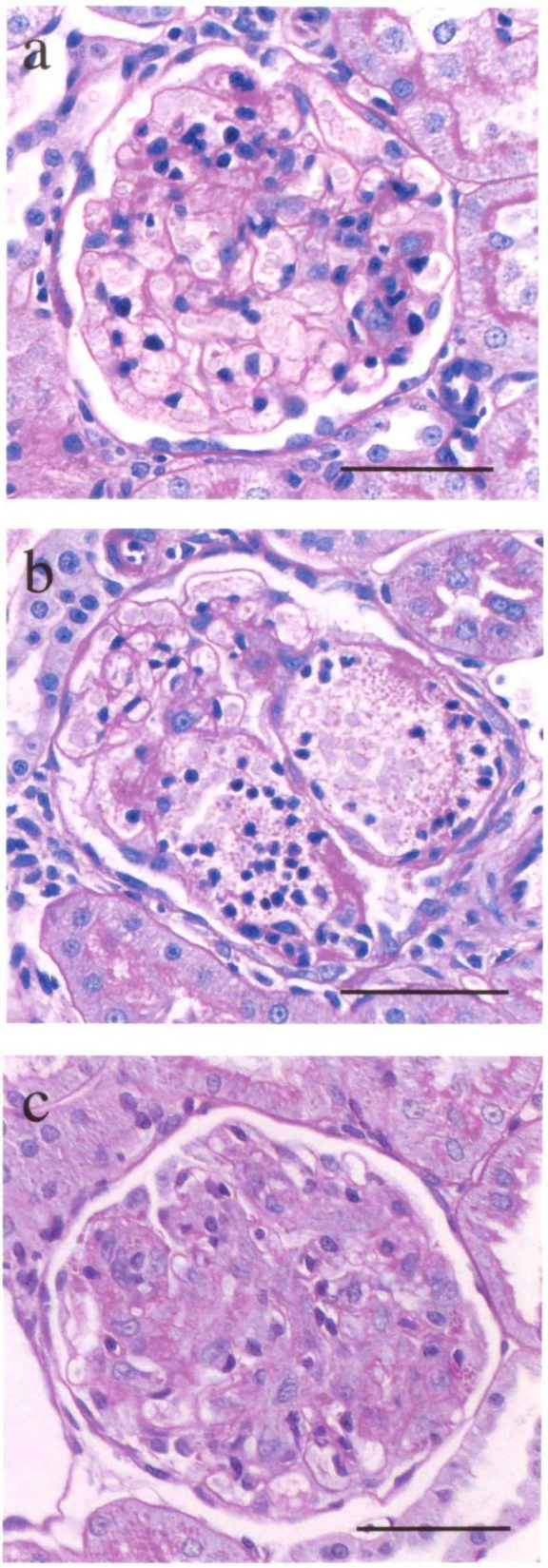
Fig. 8: Quantitative analysis of flk-1 mRNA expression in the glomeruli.

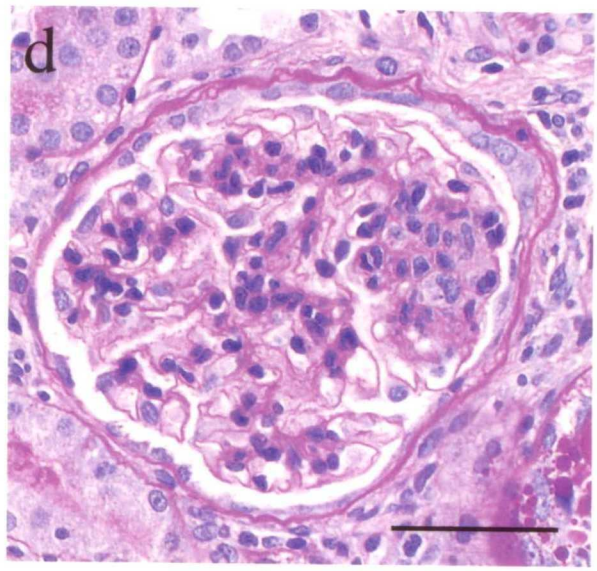
The level of flk-1 mRNA was reduced on day 3 and reached minimum on day 7 and then gradually increased. The level became significantly high on day 28 and then returned to the control level on day 56. Significant difference (＊) between nephritic and control rats was observed on days 7 and 28 ($p<0.05$). There were also significant differences (※) between each anti-Thy-1 groups from day 3 to day 28 ($p<0.05$).

Fig. 9: Localization of VEGF and flk-1 mRNA in anti-Thy-1 nephritis by *in situ* hybridization.

VEGF mRNA was detected only in podocytes in both anti-Thy-1 nephritis groups and control groups throughout the experimental periods (a: control, b: day 3, c: day 7, e: day 14 in anti-Thy-1 nephritis). There was no positive signal in the proliferative foci consisting of mesangial cells on day 7 (c and d) and 14 (e). On day 3 (b), VEGF mRNA was also expressed only in the podocytes. Bar=50 μ m.

Fig. 1

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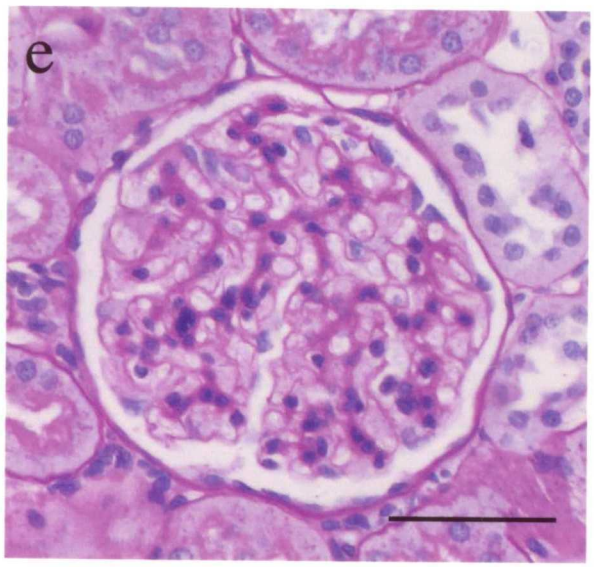
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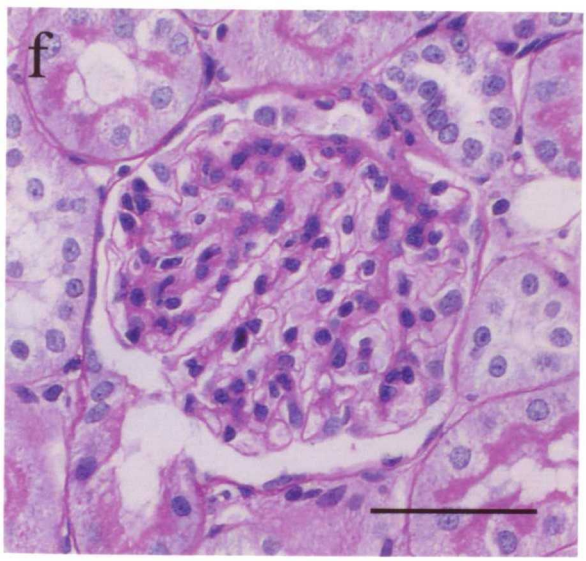
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Fig.2

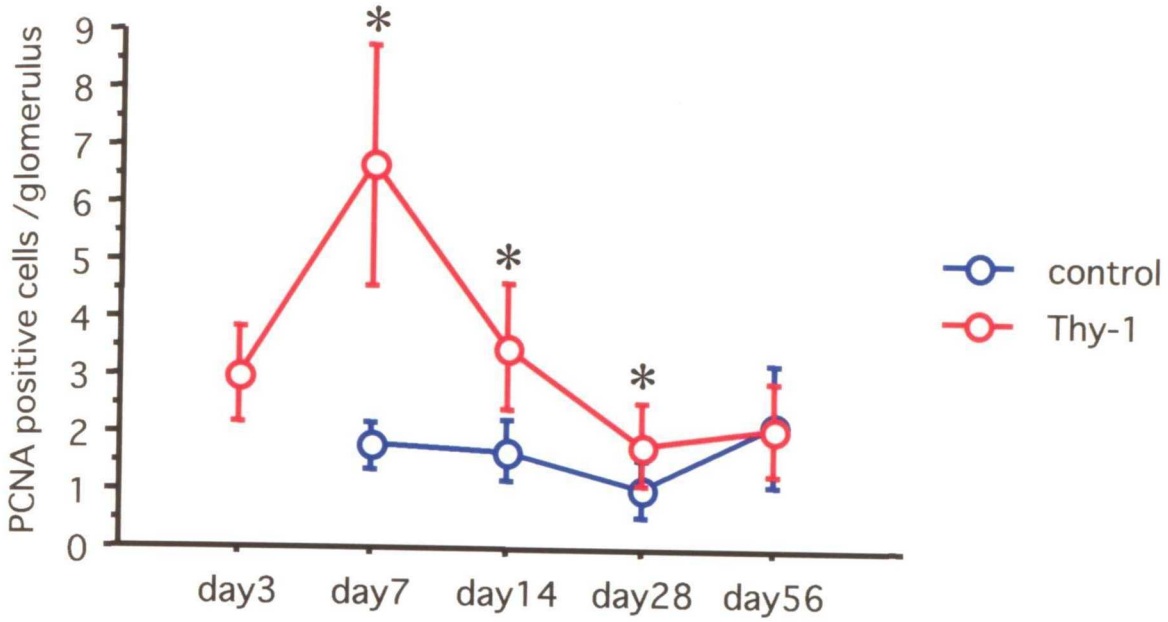
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Fig. 3

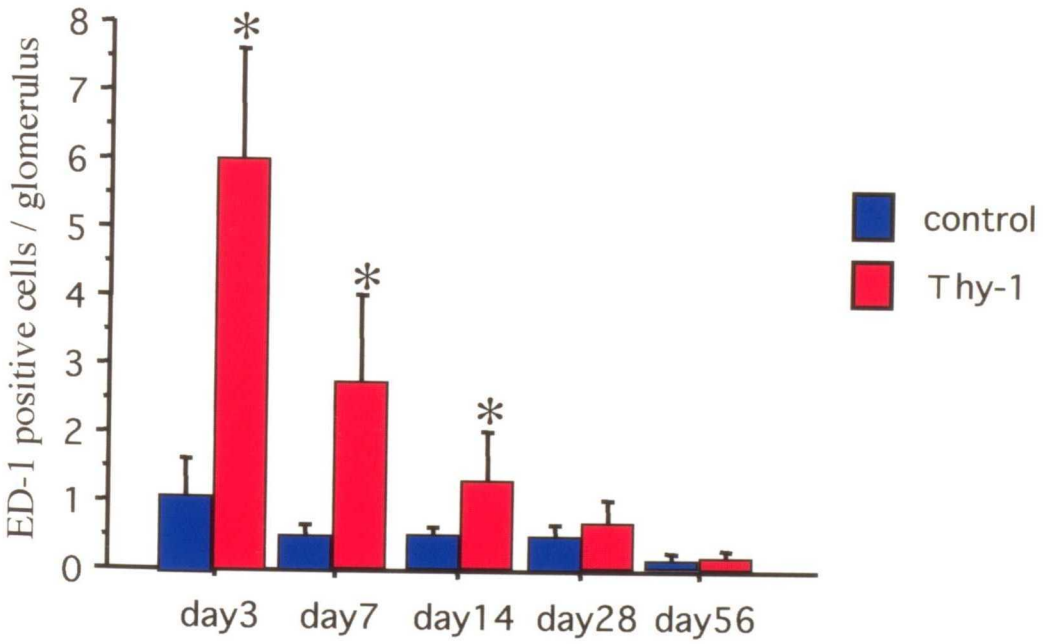
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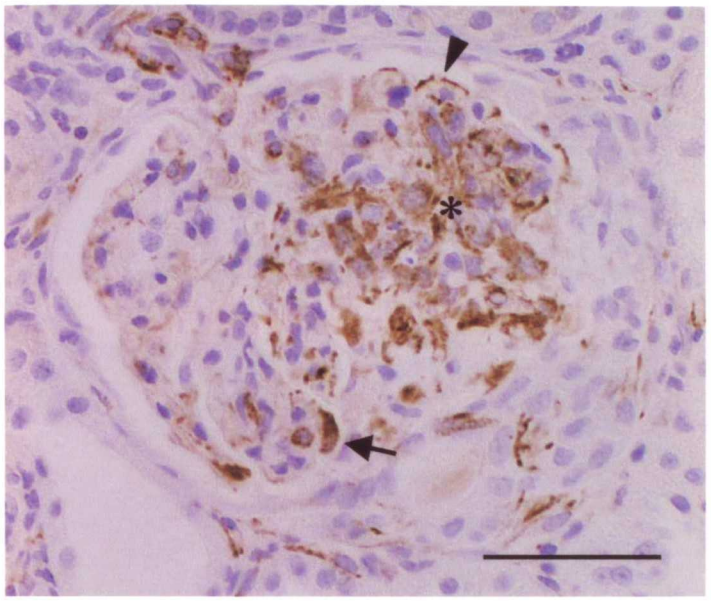
Fig. 4



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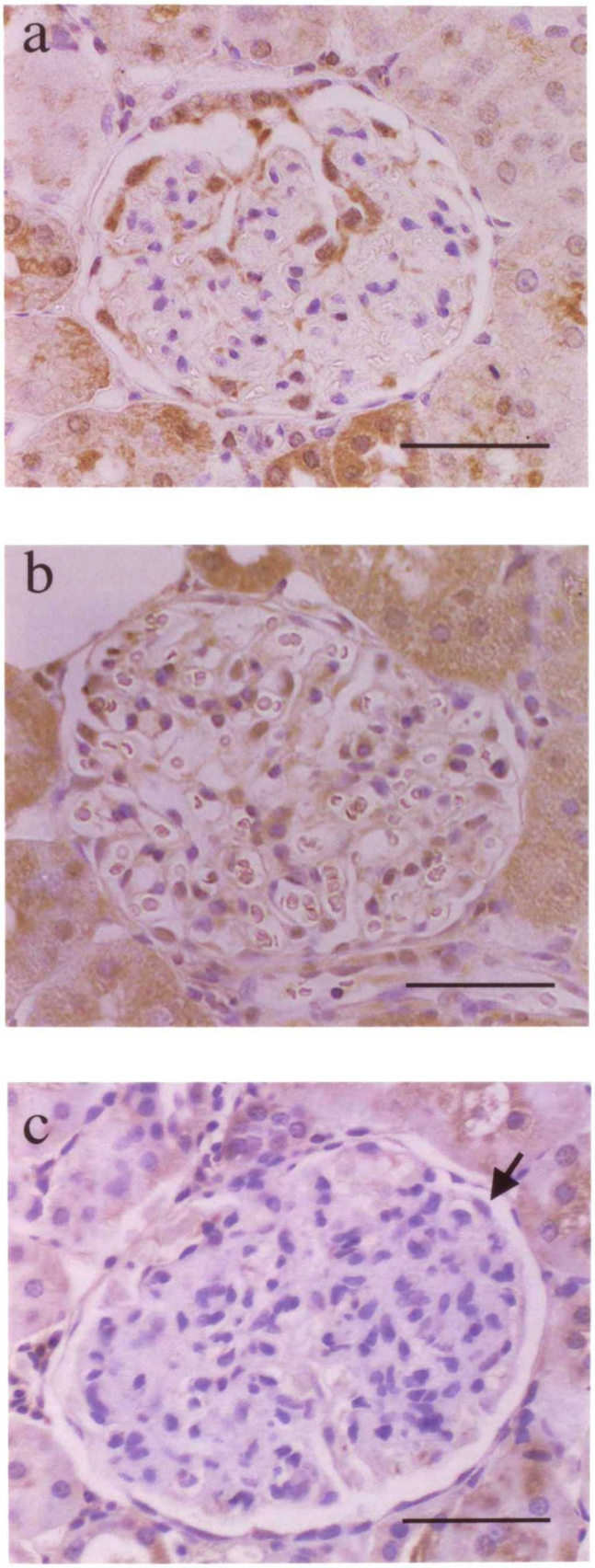

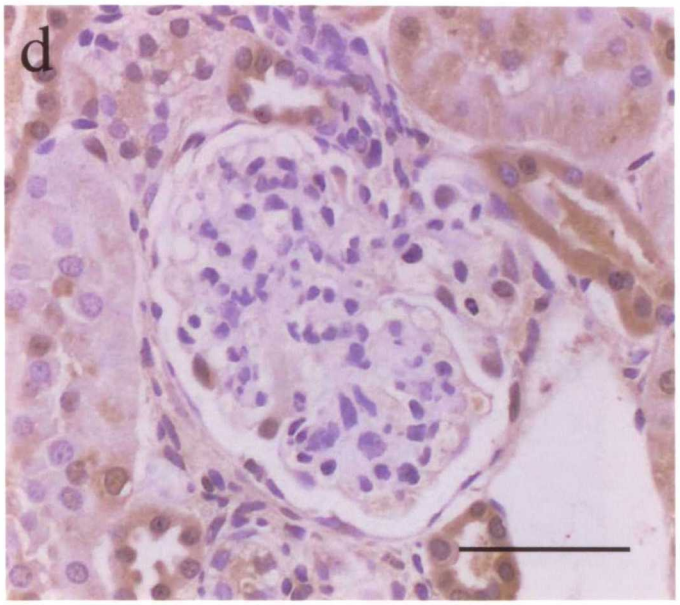
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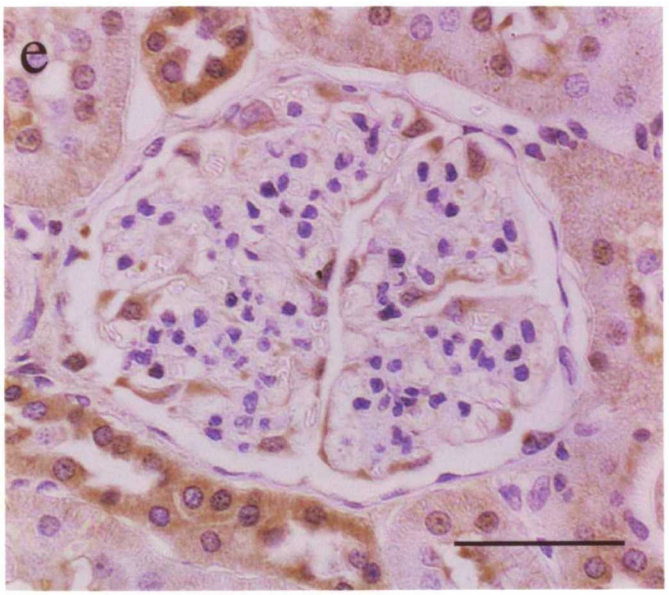
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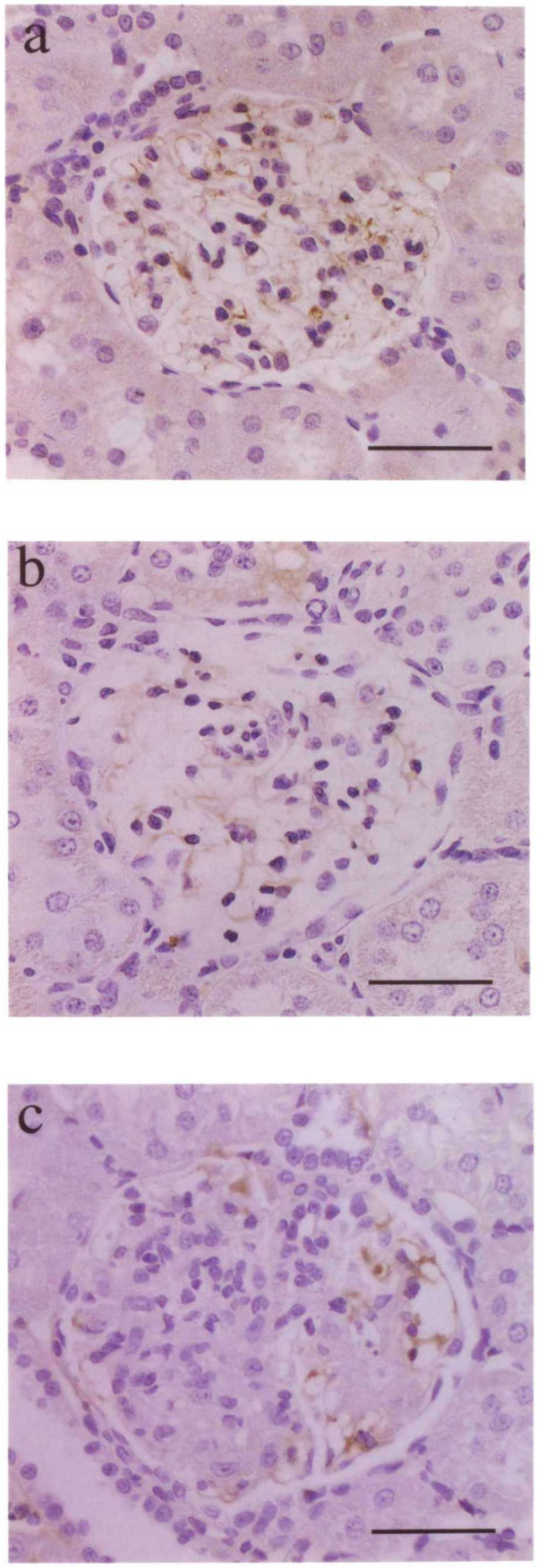
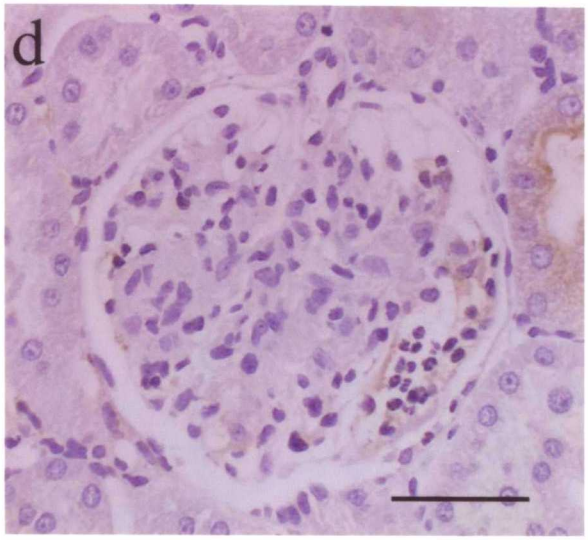
Fig. 6

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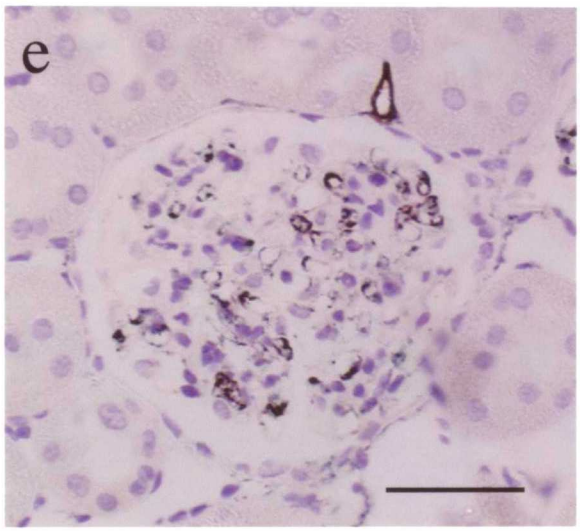
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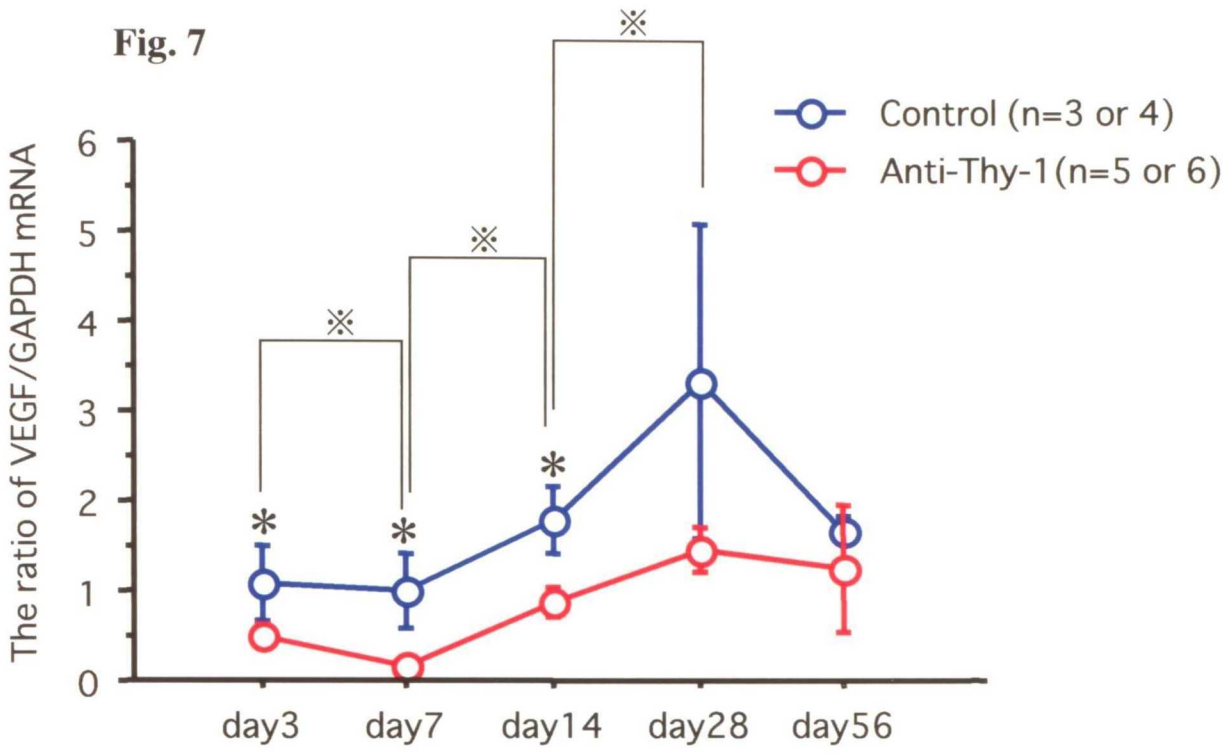
Fig. 7


The ratio of VEGF/GAPDH mRNA
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day28
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※
※
※
*
*
*
Control (n=3 or 4)
Anti-Thy-1(n=5 or 6)

Fig. 8

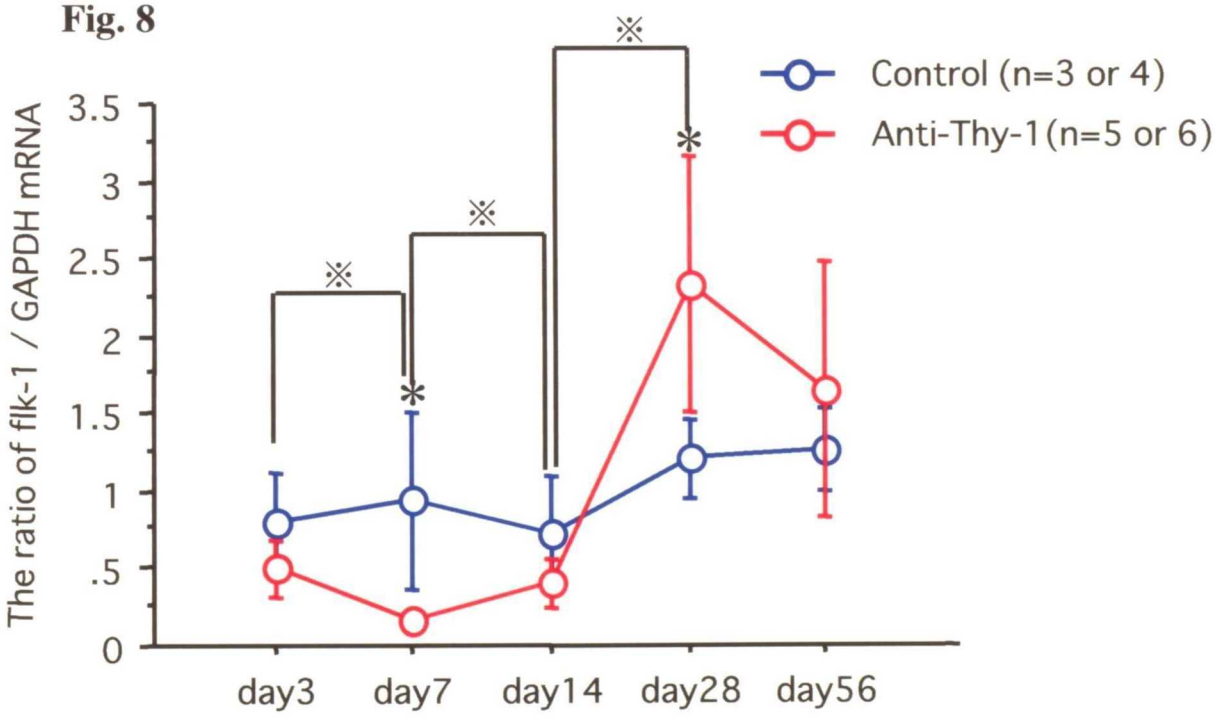
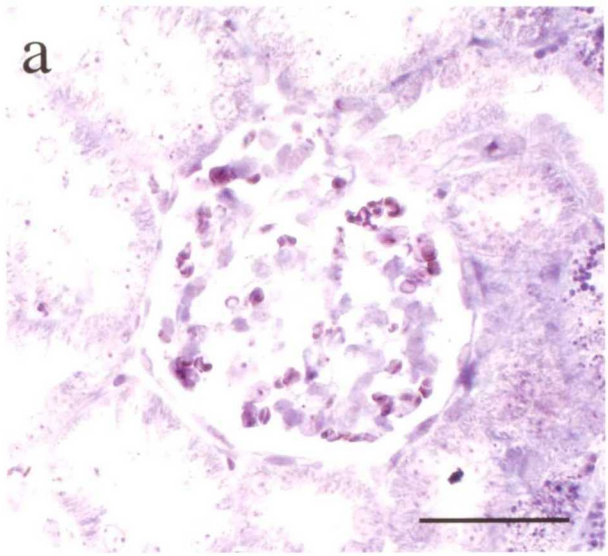
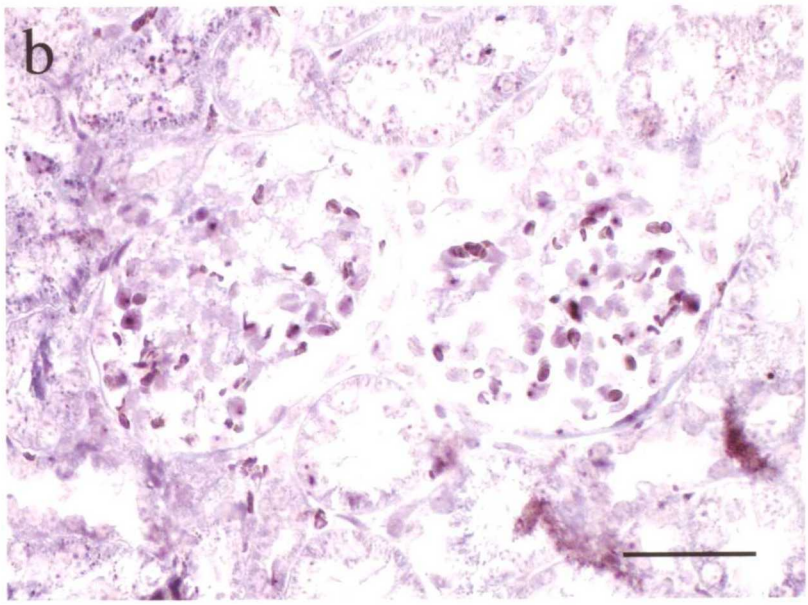
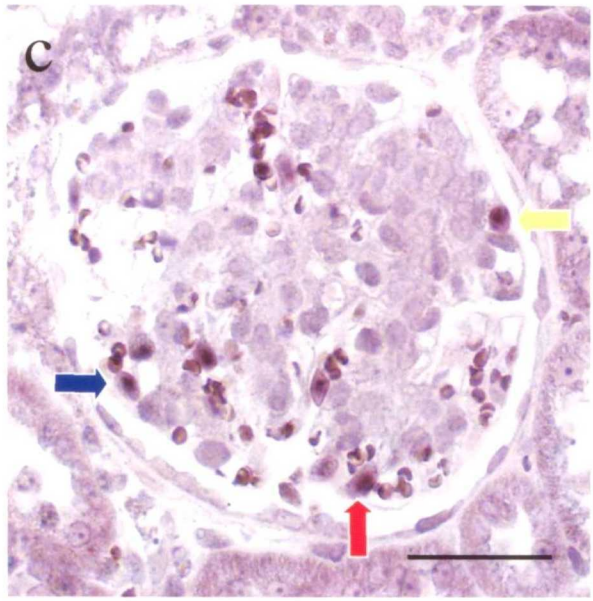

The ratio of flk-1 / GAPDH mRNA
0
.5
1
1.5
2
2.5
3
3.5
day3
day7
day14
day28
day56
※
※
※
*
*
Control (n=3 or 4)
Anti-Thy-1(n=5 or 6)

Fig. 9



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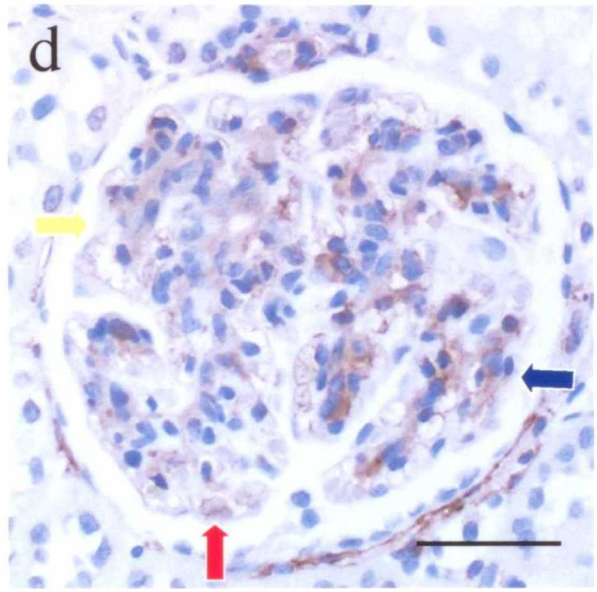
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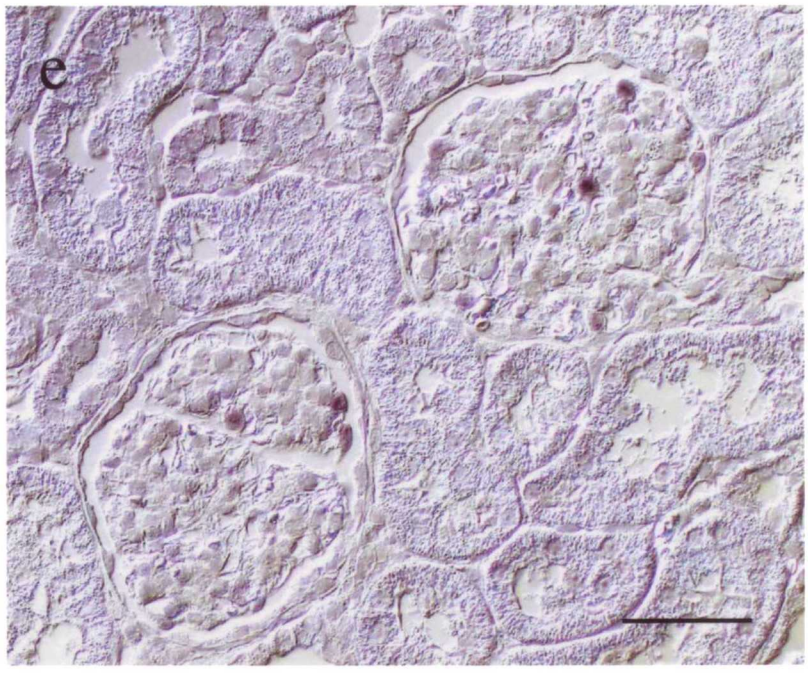
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Chapter Ⅳ

The role of fibroblast growth factor-2 (FGF-2) and its receptor on the recovery of the glomerular structure in anti-Thy-1 nephritis.

Introduction

Fibroblast growth factor-2 (FGF-2; basic FGF) belongs to a large family of fibroblast growth factors (Klagsbrun, *et al.* 1989.) and four different FGF-2 polypeptides can be formed from the one *fgf*-2 gene: in addition to the 18-kDa form, 22.5-, 23.1- and 24.2-kDa forms have also been identified (Florkiewicz, *et al.* 1989.). The 18-kDa form is a result of translational initiation at the 5' AUG start codon, while the others are a result of translation beginning at upstream, in-frame, CUG codons (Florkiewicz, *et al.* 1989. Prats, *et al* 1989.). The localization of these polypeptides was reported that the 18-kDa form remained cytoplasmic and the larger forms associated with the nucleus (Renko, *et al.* 1990.), which was due to the addition of a nuclear localization signal in the 37 amino acids upstream of the AUG start site (Bugler, *et al.* 1991.). Moreover, because FGF-2 does not contain classical leader sequences which affect the halt of translation and control the synthesis of amino acids, it may be transported to the extracellular space by the novel secrete pathway that may be independent of the endoplasmic reticulum (ER) and the Golgi apparatus. Following cellular release, FGF-2 may be sequestered in the extracellular matrix by binding to heparan sulfate proteoglycans (HSPGs) (Klagsbrun, 1992.). HSPGs in the cell membrane also act as low-affinity receptors for FGF-2 and are necessary for signaling by this peptide (Ruoslahti, *et al* .1991.). In addition to HSPG low-affinity receptors, four high-affinity FGF receptors (FGFR1 to FGFR4) have been identified. The receptors are characterized by three extracellular immunoglobulin (Ig)-like domains (designated Ig Ⅰ, Ig Ⅱ and Ig Ⅲ), a transmembrane domain and a split tyrosine kinase domain (McKeehan, *et al.* 1998.), and FGFR1 to FGFR3 have

different splice variants of IgⅢ (Ⅲb and Ⅲc variants) that have different ligand-binding affinities (Ornitz, *et al.* 1996., Xu, *et al.* 1998., Coutts, *et al.* 1995., Johnson, *et al.* 1993.).

FGF-2 is expressed in many kinds of cells including fibroblasts, endothelial cells, vascular smooth muscle cells and macrophages (Klagsbrun, *et al.* 1989.). In the kidney, FGF-2 is a potent mitogen *in vitro* (Silver, *et al.* 1988.) and is also expressed in the rat mesangial cells *in vivo* (Floege, *et al.* 1992.). Furthermore, FGF-2 exhibits mitogenic actions on other glomerular cells, such as glomerular endothelial cells and epithelial cells (podocytes) *in vitro* (Ballermann, *et al.* 1989., Takeuchi, *et al.* 1992.).

Experimental evidences suggest that FGF-2 may be involved in the induction and stimulation of mesangial proliferation in the autocrine/paracrine manner (Floege, *et al.* 1992., Floege, *et al.* 1993.). It was also suggested that increased FGF-2 concentrations in the glomerulus were insufficient to induce mesangial cell proliferation *in vivo*, and the responsiveness of mesangial cells towards FGF-2 required priming by sublethal injury (Floege, *et al.* 1992. Floege, *et al.* 1993.). It was revealed that FGF-2 might not be a significant mesangial cell chemoattractant *in vitro* and *in vivo* (Haseley, *et al.* 1999.). Apart from mesangial cell proliferation, it was shown that exogenous FGF-2 injection induced increased proteinuria, additional damage of injured podocytes and glomerulosclerosis in a model of membranous glomerulonephritis, passive Heymann nephritis (PHN) (Floege, *et al.* 1995.). Moreover, it was suggested that constitutive release of FGF-2 after immune-mediated cell injury contributed to glomerular cell damage and FGF-2 was an endogenous amplifier of cytotoxic damage following immune-mediated

injury to glomerular cells (Floege, *et al.* 1998.). Thus, FGF-2 may play an important role in the progression of glomerulonephritis.

FGF-2 is also known as one of the angiogenic factor *in vitro* and *in vivo*. It was demonstrated that capillary endothelial cells produced and released FGF-2 and it might act on endothelial cells in the autocrine mechanism *in vitro* (Schweigerer, *et al.* 1987.), and glomerular endothelial cells might be stimulated their proliferation by FGF-2 in the autocrine/paracrine mechanisms (Ballermann, *et al.* 1989.). Anti-Thy-1 nephritis is the reversible model of mesangioproliferative glomerulonephritis, which was described in chapter-3 of this thesis. In recent years, a "resolution" phase of this model has been studied to understand the mechanisms of spontaneous glomerular capillary repair after the disruption of glomerular structure, and it was indicated that the glomerular endothelial cell was an active participant in the capillary repair and restoration of glomerular architecture (Iruela-Arispe, *et al.* 1995.). It was suggested that FGF-2 would be involved in glomerular endothelial cell proliferation observed in early phase of anti-Thy-1 nephritis (Iruela-Arispe, *et al.* 1995.). However, there are a few studies on the role of FGF-2 in the recovery of injured glomeruli.

In chapter-3, the author suspected that VEGF might greatly contribute to the reconstruction of injured glomeruli to stimulate glomerular cells such as endothelial cells as well as mesangial cells. Therefore, the author performed quantitative analysis of VEGF and flk-1 mRNA in the glomeruli and determined their localization. The results of chapter-3 suggested that VEGF might play a role on the recovery of the glomerular structure. Therefore, in this chapter, the author noted FGF-2/FGF receptor (FGFR) as an another

candidate to have an important role in glomerular recovery, and examined FGF-2 and FGFR localization and FGF-2 mRNA expression by real-time PCR to clarify their role in anti-Thy-1 nephritis.

Materials and Methods

Anti-Thy-1 glomerulonephritis model on rats

For the induction of anti-Thy-1 nephritis (anti-Thy-1 nephritis group), 8-week-old male Wistar rats (Charles River Japan, Inc., Yokohama, Japan) were injected intravenously with 0.5 ml / 100g body weight of polyclonal anti-Thy-1 antibody produced in rabbits immunized with rat thymocytes as described previously (Ishizaki, *et al.* 1986.). As the control group, rats were injected with normal rabbit serum in stead of anti-Thy-1 antibody. Rats were sacrificed by bleeding under ether anesthesia on days 3, 7, 14, 28 and 56 after the administration of anti-Thy-1 antibody or normal rabbit serum (n = 4 to 6 per time point) and their kidneys were collected.

Histopathological and immunohistochemical examinations.

After removal of the kidney, renal tissues were fixed in 10% formalin neutral buffer solution, Mildform (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin. The sections (3 μm) were stained with hematoxylin and eosin (H-E) and periodic acid-Schiff (PAS) for light microscopic examination.

For immunohistochemistry of FGF-2 and FGFR, paraffin sections were used, and the specimens were stained by the streptoavidin-biotin-peroxidase complex technique using Histofine kit (Nichirei Corp., Tokyo, Japan). The following antibodies were used as primary ones: 1) monoclonal mouse anti-FGF-2 antibody (clone bFM-2, Upstate, Lake Placid, NY, USA), 2) monoclonal mouse anti-FGFR antibody (clone Ab-1, Oncogene Research Products, San Diego, CA, USA), which can detect FGFR1, 2 and 3. For the

staining of FGF-2, 4% paraformaldehyde (PFA)-fixed paraffin sections were used and pretreated with 2mg/ml hyaluronidase (Wako Pure Chemicals, Osaka, Japan) for 30 min at 37℃. For the staining of FGFR, formalin fixed paraffin sections were preincubated with pepsin solution (Nichirei Corp., Tokyo, Japan) for 20 min at 37℃. The immunoreaction was visualized by a diaminobendine-hydrogen peroxidase solution, and the sections were counterstained with hematoxylin. To analyze the condition of glomerular endothelial cells, immunofluoroscent examination was performed. The frozen sections fixed with acetone were incubated with antibodies as follows; mouse monoclonal anti-rat endothelial cells antigen (RECA)-1 antibody (Duivestin, *et al.* 1992,) as primary one, which is a surface antigen expressed on all rat endothelial cells, and FITC-conjugated affinity purified goat anti-mouse IgG antibody (EY Laboratories, San Mateo, CA) as second one. Glomerular capillaries identified by positive staining for RECA-1 were observed under a fluorescence microscope or a confocal laser-scanning microscope.

Laser microdissection, RNA extraction and reverse transcription reaction.

A part of the renal cortex of each rat was quickly frozen in liquid nitrogen and stored at –80℃ until use. Frozen sections (8 μm) of the kidneys were made and mounted on glass slides covered with PEN foil (2.5 μm thick; Leica Microsystems, Wetzlar, Germany) for the microdissection system used in this study. They were fixed with 70% ethanol for 10sec, gently washed twice with diethylpyrocarbonate (DEPC)-treated water, and thoroughly air-dried. After that, the sections were stained with 0.05% toluidine blue (TB)

solution, pH 4.1, (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10sec, and then the TB solution was rinsed with DEPC-treated water, and the sections were air dried. Then renal glomeruli were dissected from the frozen sections with the LMD system using a 337-nm nitrogen ultraviolet (UV) laser (Leica Microsystems, Wetzlar, Germany). The glomeruli dissected from a section dropped immediately into a tube cup filled with 50 μ l RNA extraction buffer. Two-hundreds glomeruli, which is the enough number to quantify mRNA expression in the glomeruli (Inoue, et al. 2003), were collected into a 0.5-ml tube. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and DNase digestion was performed with RNase-free DNase set (Qiagen). First strand cDNA was made from total RNA using Sensiscript (Qiagen) with oligo $(dT)_{15}$ primer (Promega, Madison, Wis., USA).

Real-time PCR

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). The probe and primer pair for FGF-2 was designed using Primer Express version 1.5 (Applied Biosystems) based on their sequence. The FGF-2 TaqMan probe was 5'-ACAACACTTACCGGTCACGGAAATACTCCA-3'. The FGF-2 forward primer sequence was 5'-TGAACGCCTGGAGTCCAATAAC-3', and its reverse primer sequence was 5'-CGTTTCAGTGCCACATACCAA-3'. GAPDH TaqMan probe, forward and reverse primers were obtained from TaqMan Rodent GAPDH Control Regents (Applied Biosystems). The PCR reaction mixture consisted of distilled water (D. W.), TaqMan probes and

primers of GAPDH or FGF-2, TaqMan Universal PCR Master Mix (Applied Biosystems) and the cDNA from the dissected glomeruli which was same sample used for the analysis of VEGF and flk-1 in chapter-3. PCR conditions were as follows: 2 min at 50℃ for uracil-N-glycosylase incubation, 10 min at 95℃ for the activation of AmpliTaq Gold DNA polymerase, and then 50 cycles of 95℃ for 15 sec and 60℃ for 1 min. The amplification plots of FGF-2 and GAPDH in each sample was analyzed on the point of threshold cycle number, and the relative quantity of GAPDH and FGF-2 was analyzed using the Comparative Ct Method described in User Bulletin＃2: ABI PRISM 7700 Sequence Detection System.

Statistical analysis

Data were expressed as mean±SD. Statistical significance was evaluated by the Mann-Whitney U-test. A value of $P<0.05$ was taken to denote statistical significance.

Results

Histopathological examination of anti-Thy-1 nephritis.

The histopathological findings were same as those in chapter-3. In anti-Thy-1 nephritis, mesangial lesions including mesangiolysis were observed on day 3 and the glomeruli on day 7 showed microaneurysm and intensive diffuse or segmental cell proliferation. Proliferating lesions remained in some glomeruli although some of them were being repaired on day 14. Almost glomeruli had been repaired by day 56.

The changes of glomerular endothelial cells in anti-Thy-1 nephritis.

RECA-1 positive glomerular endothelial cells decreased from day 3 accompanied with destruction of glomerular structure (Fig.1a). In the proliferating foci, there were no RECA-1 positive cells and they could be observed remaining capillaries in the glomeruli of days 7 and 14 (Fig. 1b and c). The number of RECA-1 positive cells distinctly increased and lots of small capillary lumens were observed from day 28 (Fig. 1d). On day 56, the capillary structure was almost repaired and many RECA-1 positive endothelial cells and capillary lumens were observed (Fig. 1e). However, the intensity of RECA-1 positive signals was weaker than that of control group (Fig. 1f).

Localization of FGF-2 and FGFR in the glomeruli of anti-Thy-1 nephritis.

FGF-2 was observed in glomerular endothelial cells outlined the opened capillaries and in some podocytes (Fig. 2b) in anti-Thy-1 nephritis (Figs. 2a to d). There were no positive cells in the proliferating foci in the glomeruli on

days 7 and 14 (Fig. 2c). FGF-2 was also positive in endothelial cells control groups (Fig. 2a).

FGFR was located only in endothelial cells in control and anti-Thy-1 nephritis groups (Figs. 3a to d). There were no positive cells in the proliferating foci on days 7 and 14 (Fig. 3c).

Quantitative analysis of FGF-2 mRNA expression in the glomeruli.

In anti-Thy-1 nephritis, FGF-2 mRNA level was minimum on day 3 and then significantly increased and returned to the control level by day 14 (Fig.4). Significant differences between nephritic and control rat was observed on days 3 and 7. ($p<0.05$). There was also significant difference between days 3 and 7 in anti-Thy-1 nephritis ($p<0.05$).

Discussion

In this study, the author have demonstrated that both FGF-2 and FGFR were located in glomerular endothelial cells and they were not detected in proliferating foci on day 7 and 14 in anti-Thy-1 nephritis. FGF-2 was also detected in some podocytes in anti-Thy-1 nephritis. Some studies have demonstrated the localization of FGF-2 and/or FGFRs in the kidneys of normal adult human and rat by immunohistochemistry and/or *in situ* hybridization (Cancilla, *et al.* 2001., Floege, *et al.* 1999.). In human glomeruli, FGF-2 was detected in mesangial cells, endothelial cells, podocytes, parietal epithelial cells and circulating or infiltrating leukocytes by immunohistochemistry using four different primary antibodies. As regards FGFR-1, most glomerular cells were unreactive with two antibodies by

immunohistochemistry in normal human glomeruli (Floege, *et al.* 1999.). In a normal rat, both FGFR-1 and FGFR-3 were detected in the podocytes and the parietal epithelial cells, and FGFR-3 was also detected in several glomerular cell types which might be mesangial cells and/or endothelial cells by immunohistochemistry (Cancilla, *et al.* 2001.). In anti-Thy-1 nephritis, it was reported that FGF-2 was detected in mesangial cells by immunohistochemistry (Floege, *et al.* 1992.) The immunohistochemical results of this study were different from these reports. In the case of FGF-2, it has been reported that fixation conditions have a major influence on the apparent localization of the cytokine within rat kidney (Eckenstein, *et al.* 1991.). In addition, four different anti-FGF-2 antibodies detected different expression pattern in normal human glomeruli (Floege, *et al.* 1999.). Thus, it may be difficult to localize FGF-2 protein in the glomerulus exactly by immunohistochemistry.

FGF-2 is known as one of the angiogenic factors which act on endothelial cells in the autocrine/paracrine mechanism (Schweigerer, *et al.* 1987., Ballermann, *et al.* 1989.). In anti-Thy-1 nephritis, it was indicated that the glomerular endothelial cell was an active participant in the capillary repair and restoration of glomerular architecture (Iruela-Arispe, *et al.* 1995.). It was also indicated that the number and density of glomerular endothelial cells have been shown to decrease (Wada, *et al.* 2002.). Moreover, it was suggested that FGF-2 would be involved in glomerular endothelial cell proliferation observed in early phase of anti-Thy-1 nephritis (Iruela-Arispe, *et al.* 1995.). In this study, RECA-1 positive glomerular endothelial cells distinctly increased in the resolution phase compared to the proliferating phase, though the

number of RECA-1 positive cells was not counted. This observation indicated that glomerular endothelial cells would proliferate after glomerular injury. As regards the level of FGF-2 mRNA in the glomeruli, it was minimum on day 3 and then gradually increased and reached to the control level by day 14 accompanying with endothelial cell proliferation. Moreover, both FGF-2 and FGFR were expressed in glomerular endothelial cells, suggesting autocrine mechanism would exist in these cells, and FGF-2 also located in the podocytes, suggesting that paracrine mechanism would also work on endothelial cells.

Experimental evidences in anti-Thy-1 nephritis suggested that FGF-2 might be an important factor of mesangial cell proliferation (Floege, *et al.* 1992., Floege, *et al.* 1993.). However, FGF-2 and FGFR could not be detected in mesangial proliferating lesions on day 7 and 14, and the level of FGF-2 mRNA was lower than control group in proliferative phase in this study. Previous study has reported that FGF-2 and FGFR-1 to –4 mRNA expressions in the mesangial areas were upregulated during the mesangial proliferative phase, which were examined by in situ hybridization (Jyo-Oshiro, *et al.* 1999.). In this study, the localization of these mRNA was not analyzed but there is a little possibility which proliferating mesangial cells produce FGF-2 and/or FGFR.

Following cellular release, FGF-2 may be sequestered in the extracellular matrix by binding to heparan sulfate proteoglycans (HSPGs) (Klagsbrun, 1992.). Glypican-1 is the prototype member of the glycosylphosphatidilinositol anchored cell surface HSPGs (David, 1993.), and the only member that is expressed in the vascular system (Rosenberg, *et al.*

1997., Mertens, *et al.* 1992.). It was suggested that glypican-1 on the surface of endothelial cells may act to shuttle VEGF164 produced by podocytes across the GBM and could mediate VEGF164 to contact and activate signaling flk-1 (Gengrinovitch, *et al.* 1999., Kang, *et al.* 2002.). Similarly, FGF-2 produced by glomerular cells might act on FGFR in the glomerular endothelial cells through extracellular matrix (ECM) including Glypican-1.

It was reported that endothelial cell spreading or elongation increases sensitivity to specific growth factor, such as FGF-2 *in vitro* (Ingber, *et al.* 1991.). It is unclear whether this phenomenon is operating *in vivo*. In the minireview (Folkman, *et al.* 1992.), it was discussed that in early phase of angiogenesis, because vasodilation of the parent venule occurred before the emergence of the first capillary sprout, endothelial cells might be stretched so that they become responsive to FGF-2 or other growth factors. It was reported that FGFs were stored not only in the extracellular matrix, but also in endothelial cells and various growth factors including FGF-2 were released from endothelial cells by mechanical force (McNeil, *et al.* 1989.). In early phase of anti-Thy-1 nephritis, mild expansion of capillary and microaneurysm are observed and endothelial cells may be elongated by the expansion of capillary lumen and get injured by formation of mesangial lesions. The previous report suggested that FGF-2 might act on endothelial cell proliferation in early phase of this model (Iruela-Arispe, *et al.* 1995.). Therefore, the sensitivity of endothelial cells to FGF-2 might increase in the early phase and FGF-2 released from endothelial cells and other glomerular cells would act on FGFR in endothelial cells in anti-Thy-1 nephritis.

In this study, the author demonstrated FGF-2 and FGFR localization and

FGF-2 mRNA expression in the glomeruli of anti-Thy-1 nephritis and suggested the possible role of FGF-2 and FGFR on glomerular reconstruction by autocrine manner in the endothelial cells and by paracrine manner between the podocytes and endothelial cells. Many factors such as FGF-binding protein (FGF-BP) and Cyr61, which is expressed in the podocyte in anti-Thy-1 nephritis (Sawai, *et al.* 2003.), might be concerned with modulation of FGF-2 biological activity (Tassi, *et al.* 2001, Czubayko, *et al.* 1997., Kolesnikova, *et al.* 1998.). Therefore, further studies are needed to understand the role of FGF-2 and FGFR on glomerular reconstruction.

Abstract

Fibroblast growth factor-2 (FGF-2) belongs to a large family of fibroblast growth factors and there are four different forms (18-, 22.5-, 23.1- and 24.2-kDa). FGF-2 is expressed in many kinds of cells including fibroblasts, endothelial cells, vascular smooth muscle cells and macrophages. In the kidney, FGF-2 exhibits mitogenic actions on glomerular cells *in vitro* and may be involved in many phenomenons including mesangial cell proliferation, proteinuria, glomerulosclerosis and cytotoxic damage *in vivo*. FGF-2 is also known as the angiogenic factor and it might stimulate the proliferation of glomerular endothelial cells *in vitro*. Anti-Thy-1 nephritis is the reversible model of mesangioproliferative glomerulonephritis and in a "resolution" phase of this model, glomerular endothelial cells might be active participants in the capillary repair and restoration of glomerular architecture. Recent studies have suggested that VEGF would play an important role in endothelial cell proliferation and capillary repair in anti-Thy-1 nephritis. However, the author suggested that endogenous VEGF might have little importance on the recovery of the glomerular structure in chapter-3. Therefore, in this chapter, the author selected another angiogenic factor, FGF-2 and FGF receptor (FGFR) and examined their localization and FGF-2 mRNA expression by real-time PCR to clarify their role in anti-Thy-1 nephritis because there are a few studies on the role of FGF-2 in the recovery of injured glomeruli.

Anti-Thy-1 nephritis was induced in 8-week-old male Wistar rats and their kidneys were collected on days 3, 7, 14, 28 and 56 after the administration of anti-Thy-1 antibody. Histopathological and immunohistochemical examinations for FGF-2, FGFR and RECA-1 were performed. For

quantitative analysis of FGF-2 mRNA in the glomeruli, 200 glomeruli were obtained using LMD system and total RNA was extracted followed by reverse transcript (RT) reaction to make first strand cDNA, and real-time PCR was performed using primer pair and TaqMan probe for FGF-2.

In anti-Thy-1 nephritis, mesangial lesions including mesangiolysis were observed on day 3 and the glomeruli on day 7 showed microaneurysm and intensive mesangial cell proliferation, and almost glomeruli had been reconstructed by day 56. RECA-1 positive cells decreased from day 3 and there were no RECA-1 positive cells in the proliferating foci on days 7 and 14. The number of RECA-1 positive cells distinctly increased and many RECA-1 positive cells and capillary lumens were observed by day 56. FGF-2 was observed in glomerular endothelial cells outlined the opened capillaries and in some podocytes. FGFR was located only in endothelial cells. There were no FGF-2 and FGFR positive cells in the proliferating foci in the glomeruli on days 7 and 14. FGF-2 mRNA level was minimum on day 3 and then gradually increased and became to be control levels as early as on day 14. These results suggest that FGF-2 expressed in endothelial cells and the podocytes might act on FGFR in endothelial cells. They might be greatly involved in the recovery of glomerular structure in autocrine/paracrine manner. Moreover, there might be little possibility which proliferating mesangial cells produce FGF-2 and play an important role on the proliferation of endothelial cells as well as mesangial cells.

Figure Legends

Fig. 1: Immunofluorescent microscopy on the glomerular endothelial cells in anti-Thy-1 nephritis.

RECA-1 positive glomerular endothelial cells decreased from day 3 accompanied with destruction of glomerular structure (a). In the proliferating foci, there were no RECA-1 positive cells and they could be observed remaining capillaries in the glomeruli of days 7 and 14 (b and c). The number of RECA-1 positive cells distinctly increased and many small capillary lumens were observed from day 28 (d). On day 56, the capillary structure was almost repaired and many RECA-1 positive endothelial cells and capillary lumens were observed (e). However, the intensity of RECA-1 positive signals was weaker than that of control group (f).

Fig. 2: Immunohistochemistry of FGF-2 in anti-Thy-1 nephritis.

FGF-2 was observed in glomerular endothelial cells outlined the opened capillaries and some podocytes (b, arrows) in anti-Thy-1 nephritis (b～d,b: day 7, c: day 14, d: day 28). There were no positive cells in the proliferating foci on days 7 and 14 (b and c, ＊). FGF-2 expression in endothelial cells could be also observed in control groups (a). Bar=50 μ m.

Fig. 3: Immunohistochemistry of FGFR in anti-Thy-1 nephritis.

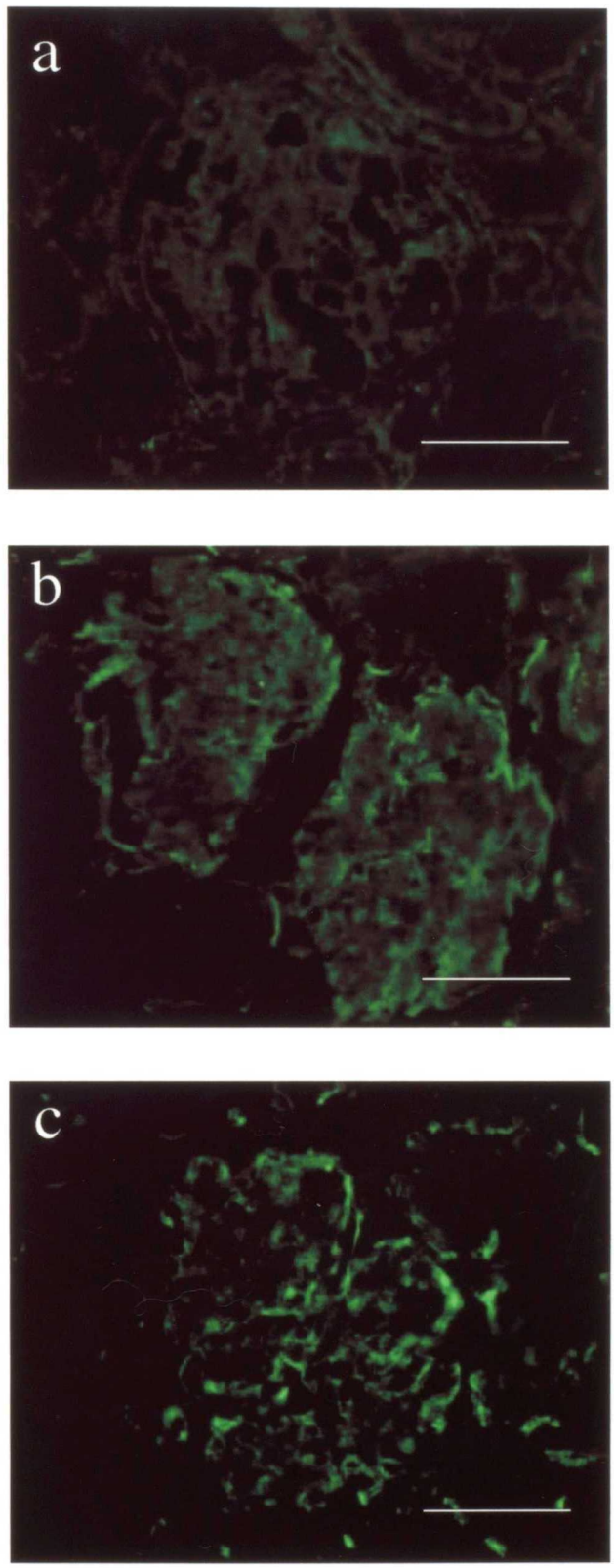
FGFR was located only in endothelial cells in control and anti-Thy-1 nephritis groups (a: control, b: day 3, c: day 7, d: day 56 in anti-Thy-1 nephritis). There were no positive cells in the proliferating foci on days 7 and

14 (c, ＊). Bar=50 μ m.

Fig. 4: Quantitative analysis of FGF-2 mRNA expression in the glomeruli.

In anti-Thy-1 nephritis, FGF-2 mRNA level was minimum on day 3 and then gradually increased and became to be same level as control groups after day 14. Significant differences between nephritic and control rats were observed on days 3 and 7 (＊,$p<0.05$). There was also significant difference between days 3 and 7 in anti-Thy-1 nephritis (※, $p<0.05$).

Fig. 1



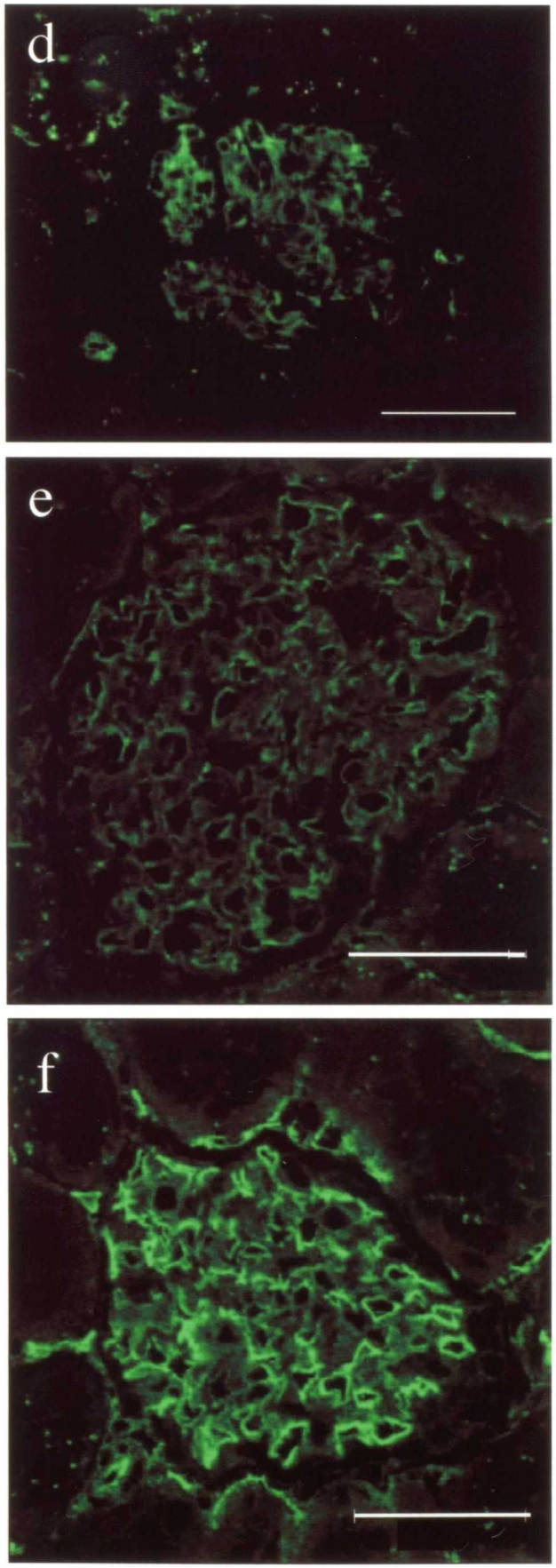

d
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Fig. 2

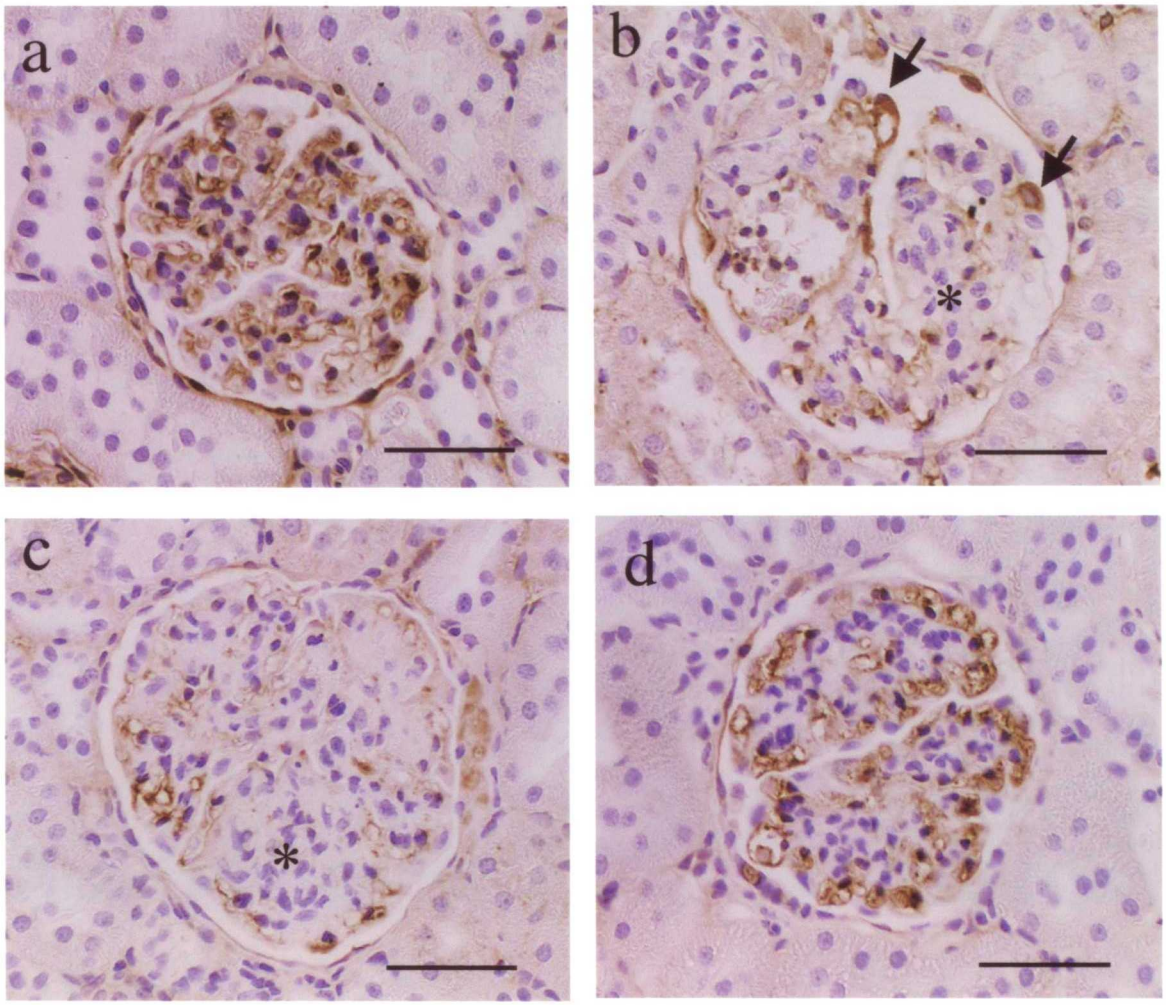


Fig. 3

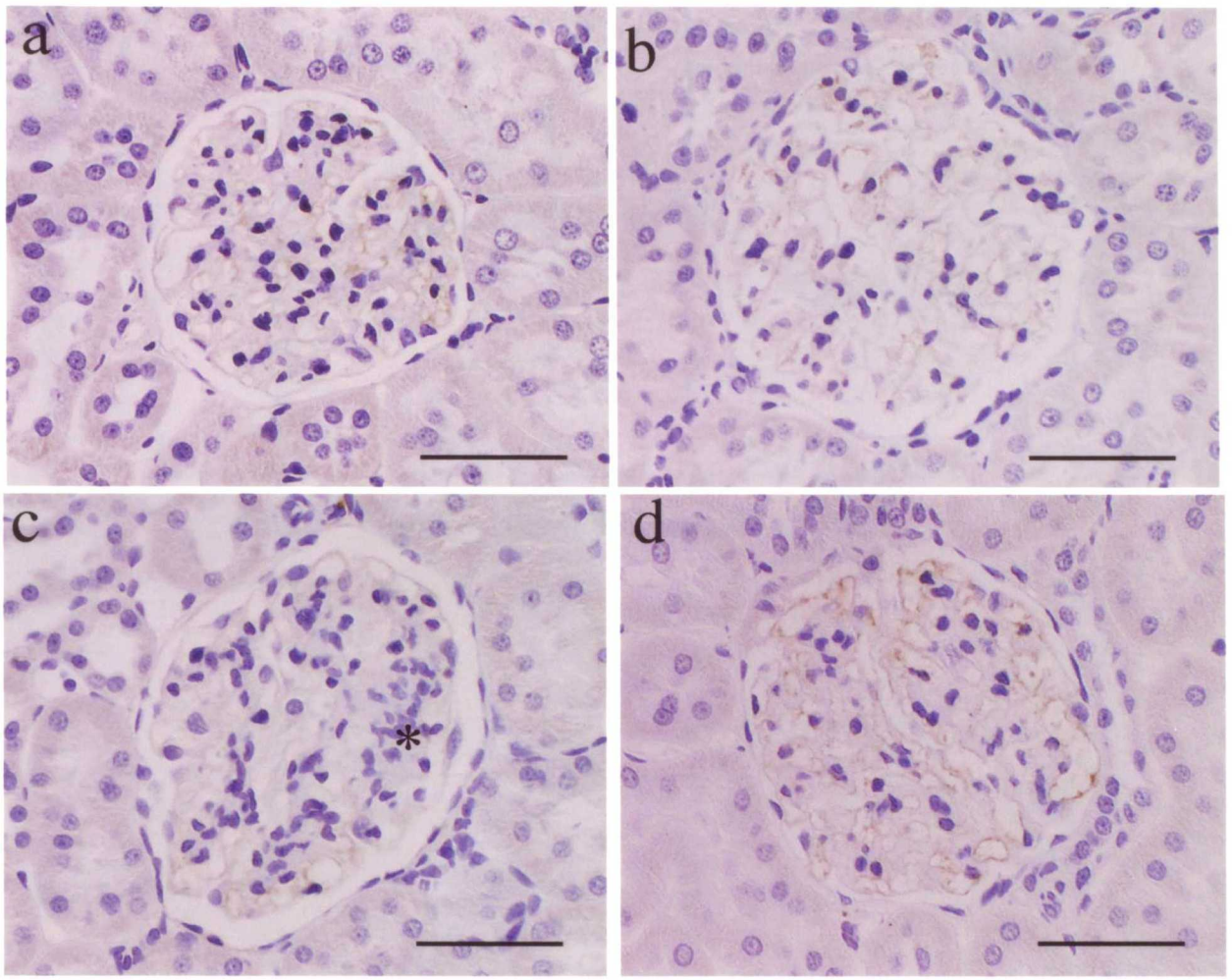
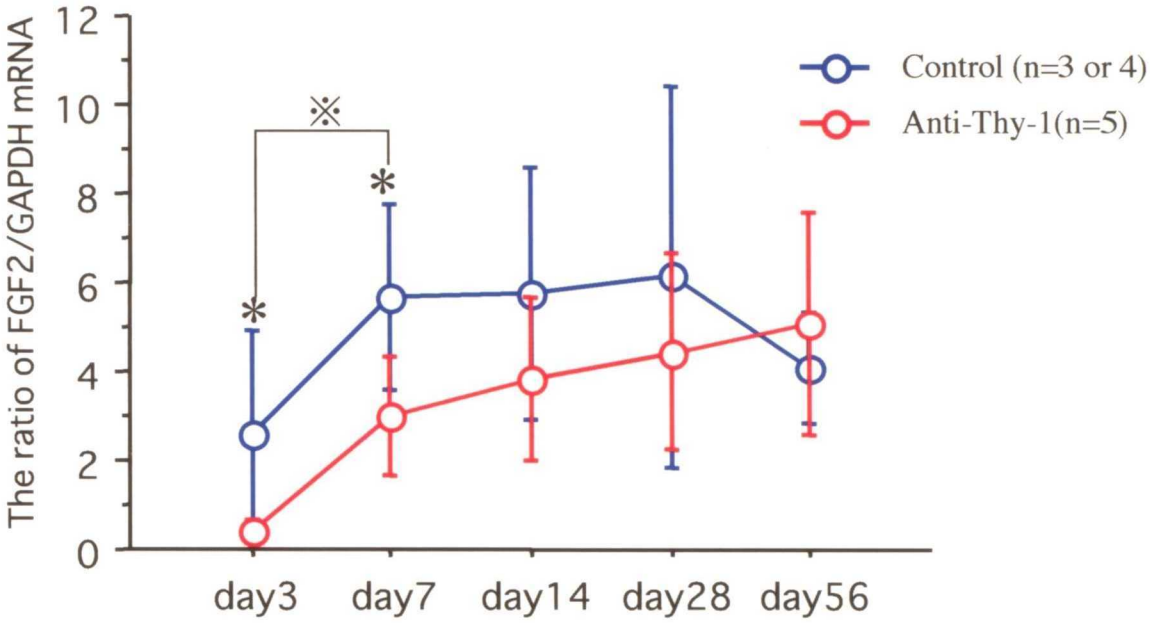


Fig. 4


The ratio of FGF2/GAPDH mRNA
12
10
8
6
4
2
0
※
*
*
day3
day7
day14
day28
day56
Control (n=3 or 4)
Anti-Thy-1(n=5)

Conclusions

The author established the methods for isolation of the renal glomeruli and for the quantitative analysis of local gene expression in the glomeruli using laser microdissection (LMD) and real-time PCR. By these methods, the author examined the role of VEGF, FGF-2 and their specific receptors in the reconstruction of injured glomeruli in anti-Thy-1 nephritis and first demonstrated that the gene expression of VEGF/flk-1 and FGF-2 was accompanied with the morphological changes in this model. As a result, the author concluded as follows.

1. The best method to isolate renal glomeruli for the quantitative analysis of local gene expression was LMD. This method could dissect glomeruli from the frozen sections exactly without contamination of other renal components and certainly enable us RT-PCR analysis with small number of dissected glomeruli.

2. To obtain the reproducible quantitative data of VEGF, flk-1 and GAPDH mRNA expression in the glomeruli, more than 100 dissected glomeruli were required using LMD system. For the analysis of flk-1, 200 glomeruli were required because of its lower gene expression in the glomeruli. The plots of cDNA derived from these glomeruli showed stable and exponential amplification by real-time PCR.

3. There may be little possibility of autocrine mechanisms of VEGF/flk-1 in

rat mesangial cells *in vivo*. VEGF and flk-1 did not localize in the mesangial proliferative foci and the levels of their mRNA were minimum in proliferative phase in anti-Thy-1 nephritis.

4. VEGF may play a role on the recovery of glomerular structure in anti-Thy-1 nephritis by paracrine manner between the podocytes and endothelial cells, because the levels of VEGF and flk-1 mRNA were gradually increased accompanying with glomerular reconstruction.

5. FGF-2 and FGFR may act on glomerular endothelial cells by autocrine/paracrine manner and play a role on the recovery of glomerular structure in anti-Thy-1 nephritis, however their role on the mesangial proliferation in injured glomeruli was not of importance in rat anti-Thy-1 nephritis.

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