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PROTEIN TYROSINE KINASES EXPRESSED IN GLOMERULI AND CULTURED GLOMERULAR CELLS: FLT-1 AND VEGF EXPRESSION IN RENAL MESANGIAL CELLS

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Summary: Protein tyrosine kinases play an important role in cellular proliferation and differentiation of various cell types. To identify potential tyrosine kinases involved in glomerular functions we have utilized the polymerase chain reaction (PCR) and degenerate oligonucleotides for isolation of such genes from isolated glomeruli, cultured mesangial cell, and glomerular endothelial cells. Sequence analysis of PCR-amplified cDNAs resulted in the isolation of 24 tyrosine kinases. Here we describe for the first time the constitutive expression of 15 tyrosine kinases, tyro-1, tyro-4, tyro-6, hyk, Ptk-3, Ryk, tie, yes, lyn, tec, Jak1, Jak2, Jak3, c-abl, and flk, in renal glomeruli. In addition, Flt-1, an endothelial cell-specific receptor for vascular endothelial growth factor (VEGF), is expressed in renal mesangial cells and its gene expression is up-regulated upon the stimulation of platelet-derived growth factor (PDGF) with the concomitant up-regulation of VEGF. These data suggest the possible involvement of VEGF/Flt-1 system in cytokine-induced mesangial cell proliferations.

Protein tyrosine kinases (PTKs) play a pivotal role in the regulation of cellular proliferation and differentiation. PTKs are involved in the signal pathways which transduce the external stimuli to cytoplasm across the cell membrane. Recently, these pathways have been extensively investigated, revealing that PTKs phosphorylate cytoplasmic substrate to transmit the signals to nucleus for the activations of the specific gene expression (1-3). Recent progress in PCR technology enabled us to investigate the

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expression of potential tyrosine kinases in any tissue or any cultured cell. Several tissues including nervous system, lung, embryoid body, or hematopoietic stem cells have been investigated for potential tyrosine kinases, revealing that more than 50 members of PTK genes has been isolated and characterized to date (1,4,5).

The renal glomerulus is a vascular structure specifically designed for the efficient ultrafiltration of blood and composed of three distinct cells: contractile mesangial cells, fenestrated endothelial cells, and epithelial cells with characteristic interdigitating foot processes. Accordingly, renal glomerulus is not only morphologically unique, but its development, functions, and pathology show certain characteristic and specific features. Dysfunction of the glomerular filtration results in leakage of proteins from blood into the urine and at last in renal insufficiency. In these pathological state, the glomerular cells often proliferate, modulate their behavior, or deposit abnormal substrates within the glomeruli. However, the molecular mechanisms underlying these conditions are still poorly understood. These backgrounds led us to investigate the constitutive and pathological expressions of tyrosine kinases in renal glomeruli, which relay the external stimuli to cellular responses. In this communication, we investigated the potential tyrosine kinases expressed in glomerular cells, either from isolated glomeruli or cultured glomerular cells by means of reverse transcriptase-polymerase chain reaction (RT-PCR) with degenerate primers. Here we describe for the first time the constitutive expression of 15 tyrosine kinases; tyro-1, tyro-4, tyro-6, hyk, Ptk-3, Ryk, tie, yes, lyn, tec, Jak1, Jak2, Jak3, c-abl, and flk, in renal glomeruli in addition to the previously known expression of tyrosine kinases; insulin-like growth factor receptor (IGFR), Trk B, platelet-derived growth factor receptor (PDGFR), Flt-1, Flk-1, basic fibroblast growth factor receptor (bFGFR), and Ufo. In addition, we show here for the first time that Flt-1, an endothelial cell-specific receptor for VEGF, is expressed in mesangial cells, but not expressed in cultured glomerular endothelial cells. We also demonstrated that the gene expressions of vascular endothelial growth factor (VEGF) and Flt-1 are up-regulated in response to the stimulation of platelet-derived growth factor (PDGF) in cultured mesangial cells. We discuss the possible involvement of VEGF/Flt-1 system in cytokine-induced mesangial cell proliferations.

Material and Methods

Glomerular cell cultures and isolation of rat glomeruli

Mesangial cell culture and isolation of rat glomeruli were essentially described previously (6) and bovine endothelial cells were cultured as described (7). Male Wistar rats of 8-10 weeks of age were used as the source of glomeruli and cultured mesangial cells. Glomeruli isolated from a bovine kidney were used as the source of cultured endothelial cells. Mesangial cells were identified by staining for actin and desmin, and lack of staining for cytokeratin and factor VIII (6). Endothelial cells were identified by staining for factor VIII, uptake of LDL, and ACE activities (7). We used rat mesangial cells and bovine endothelial cells between 6th and 10th passages. Mesangial cells and glomerular endothelial cells were cultured in Dulbecco's Modified Eagles' Medium (D'MEM, GIBCO) supplemented with 5% fetal calf serum.

PCR amplification of PTK genes

Messenger RNAs were prepared from isolated rat glomeruli, cultured rat mesangial cells, and cultured bovine endothelial cells as described previously (8). Degenerate

oligonucleotides, 5'-CA(T/C) CGI GA(T/C) (T/C)TI GCI (G/A)CI (C/A)G -3' and 5'-A(T/C)I CCI (T/A)(G/A)I (G/C)(T/A)C CAI AC(G/A) TC-3' deduced from the conserved amino acid sequences of PTK domain VI (HDLAAR) and domain IX (DVWSFGV) were used as primers in a PCR reactions with the first strand cDNA that were reverse-transcribed from mRNA of isolated rat glomeruli, rat mesangial cells, or bovine glomerular endothelial cells. PCR reaction was 48 cycles of denature for 45 sec at 96°C, anneal for 4 min at 50 °C and extension for 3 min at 72°C. The amplified fragment was blunted and cloned into Bluescript plasmid (Stratagene, La Jolla, CA). Double-strand plasmid sequencing was carried out in both directions using Sequenase (US Biochemical Corp.). The deduced amino acid sequences of PCR fragment were compared to known sequences in NBRF-PIR (release 39) or Swiss-Prot (release 27) protein database.

RNA blot analysis

Total RNAs were extracted from renal cell lines or tissues using the guanidine/CsCl method (9). Ten μg of total RNAs were electrophoresed on 1.2% agarose 10% (v/v) formaldehyde gel, transferred to nylon membrane (Hybond N^+; Amersham International, UK) and hybridized to ^{32}P-labeled probes. PTK probes used for RNA blot analysis were 180 bp PCR products encoding kinase domain of PTK genes. Cloned PCR products were labeled by random priming method (Boehringer Mannheim, Germany) and used as hybridization probes. Hybridization and washing were performed as described previously (8). Human VEGF probe was kindly provided by Dr. M. Ohnishi (Tokyo University). Flk-1 probe was 189 bp PCR-amplified cDNA fragment encoding Asn^{931}-Phe^{994} of rat Flk-1. Rat Flt-1 probe was kindly provided by Dr. M. Shibuya (Tokyo University). EF-1α probe was as described (10).

Cytokine stimulation

Cultured rat mesangial cells were maintained for 48 hrs in serum-free medium and then treated with 25 ng/ml PDGF-BB (Collaborative Research), 5 ng/ml porcine TGF-β (R&D Systems Inc.), 1 x 10^3 U/ml recombinant human IL-1β, or 1 x 10^3 U/ml recombinant human TNF-α for 12 hrs. Cells were harvested and total RNAs were prepared as described above. Cell proliferations were assayed by the incorporation of [^{3}H]-thymidine. Each study was performed in quadruplicate. IL-1β was kindly provided by Otsuka Pharmaceutical Co. (Tokushima, Japan) and TNF-α was kindly provided by Dr. K. Noguchi (Teikyo University, Tokyo).

Results and Discussion

Tyrosine kinase genes expressed in glomeruli and cultured glomerular cells

In this study, we sequenced 37 PTK cDNAs and identified 24 PTK genes from glomeruli and cultured glomerular cells. As shown in Table 1, 14 PTK genes were identified as members of receptor PTK, which included IGF1R and Trk B from insulin receptor subfamily; PDGF-αR, Flt-1, and Flk-1 from PDGF receptor subfamily, basic FGFR from FGF-receptor subfamily; tyro-1, tyro-4, and tyro-6 from eph subfamily, and unclassified receptor PTKs such as Ptk-3, Ufo, Ryk, hyk, and tie. Ten PTK genes were identified as members of non-receptor PTK; which included c-abl, flk and fes from abl subfamily (Table I). Among 24 tyrosine kinases identified here, the renal or glomerular expression of IGFR, Trk B, PDGFR, Flt-1, Flk-1, bFGFR, and Ufo has been documented previously (11-16). The rest of 17 PTK genes; tyro-1, tyro-4, tyro-6, abl, flk, fes, tec, Jak1, Jak2, Jak3, Ptk-3, itk, Ryk, tie, and hyk, are not known to be expressed in renal tissue and to our knowledge this is the first report to show their expressions in renal

Table 1 Summary of PTK genes detected in glomeruli, mesangial cells, and glomerular endothelial cells

PTK genes		mRNA source		
Sub family	member	glomeruli	mesangial cells	endothelial cells
Receptor type PTK family				
Insulin Receptor				
	IGF1R		1	
	Trk B	1		
PDGF Receptor				
	PDGF-αR			1
	Flt-1		1	
	Flk-1	2		
FGF Receptor				
	bFGF-R	2		
Eph/eck/elk Receptor				
	tyro-1	1		1
	tyro-4			1
	tyro-6		1	
Unclassified				
	Ufo		4	
	Ryk			2
	hyk	3		
	tie	1		
	Ptk-3			1
Total		10	7	6
Non-receptor type PTK family				
Abl				
	abl		1	
	flk	1	2	
	fes	1		
Src				
	yes		1	
	lyn			1
	tec			3
	itk	1		
Jak				
	Jak1		1	
	Jak2	1		
	Jak3		1	
Total		4	6	4

glomeruli. In addition, in the course of present investigation, we isolated and characterized a novel Jak kinase, Jak3, from mesangial cells (17).

RNA expressions of PTK genes in renal glomeruli, cultured glomerular cells, and renal tubular cells

17 PTK genes; tyro-1, tyro-4, tyro-6, abl, flk, fes, yes, lyn, tec, Jak1, Jak2, Jak3, Ptk-3, itk, Ryk, tie, and hyk, detected in RT-PCR have not yet been previously reported to be expressed in the kidney. We then investigated the gene expression of these tyrosine kinases in liver, kidney, isolated glomeruli, mesangial cells, endothelial cells, and cultured renal tubular cell lines; LLCPK1, MDBK, and MDCK by RNA blot analyses (Fig. 1 and 2).

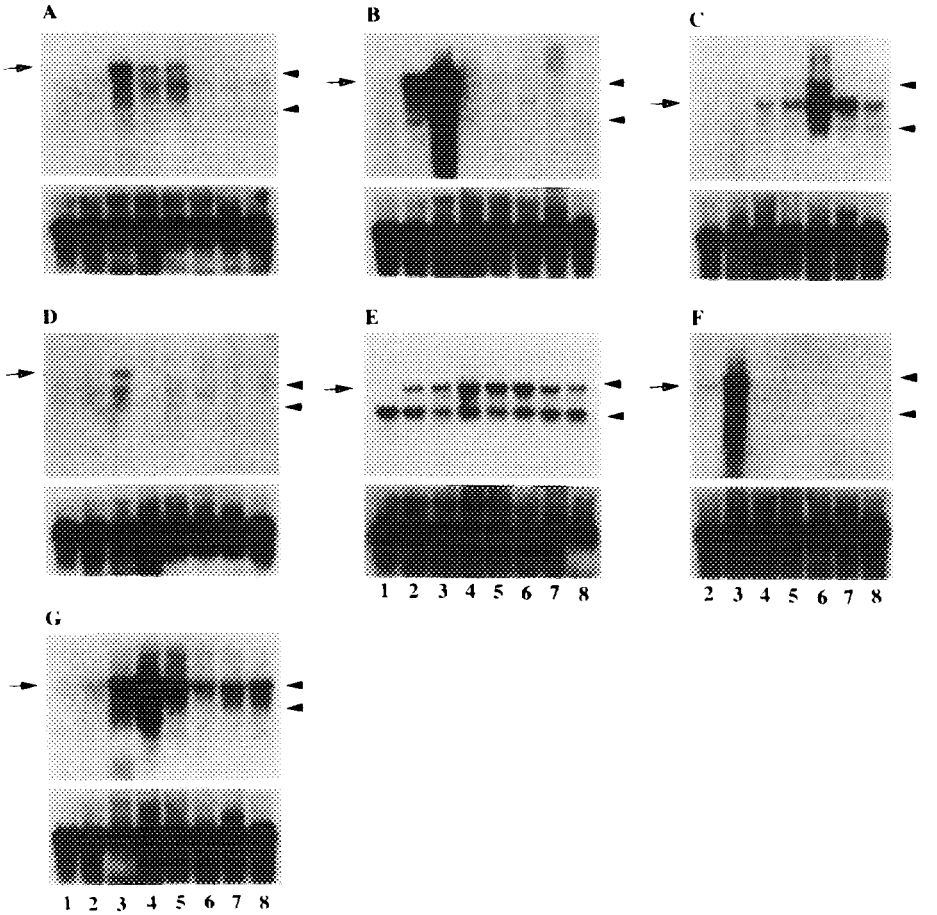

Fig. 1 Expression of receptor type PTK genes in liver, kidney, isolated glomeruli, mesangial cells, glomerular endothelial cells, and various renal tubular cell lines. Total RNAs were isolated from rat liver (lane 1), rat kidney (lane 2), isolated rat glomeruli (lane 3), rat mesangial cells (lane 4), bovine endothelial cells (lane 5), LLCPK1 (lane 6), MDCK (lane 7), and MDBK (lane 8). Ten μg of RNAs was separated on 1.2 % agarose gel containing 10% formamide and blotted onto Hybond N^+ nylon membrane. The blots were hybridized with PTK probes (A, tyro-1; B, hyk; C, Ryk; D, tyro-4; E, Ptk-3; F, tie; G, tyro-6). The blots were then hybridized with rat EF-1α (lower panel). The sizes of 28S and 18S were indicated on the right.

Among 17 kinases investigated, we failed to detect any significant signal of itk and fes (data not shown). All other 15 kinases showed, more or less, the gene expressions in renal tissues and cultured mesangial cells (Fig. 1 and 2). Among these kinases, Jak1 and Ptk-3 were ubiquitously expressed in kidney although Ptk-3 failed to show the significant signal in liver (Fig. 1E and 2D). Other tyrosine kinases showed the distinct patterns of expression in kidney: Jak3 and c-abl showed a dominant expression in mesangial cells (Fig. 2F and G) while flk, Jak2, tyro-1, and tyro-6 showed stronger signals in both mesangial and glomerular endothelial cells (Fig. 1A, G, 2E, and H). On the other hand, lyn and tec showed stronger expressions in glomerular endothelial cells and renal tubular cells but failed to show the expression in mesangial cells (Fig. 2B and

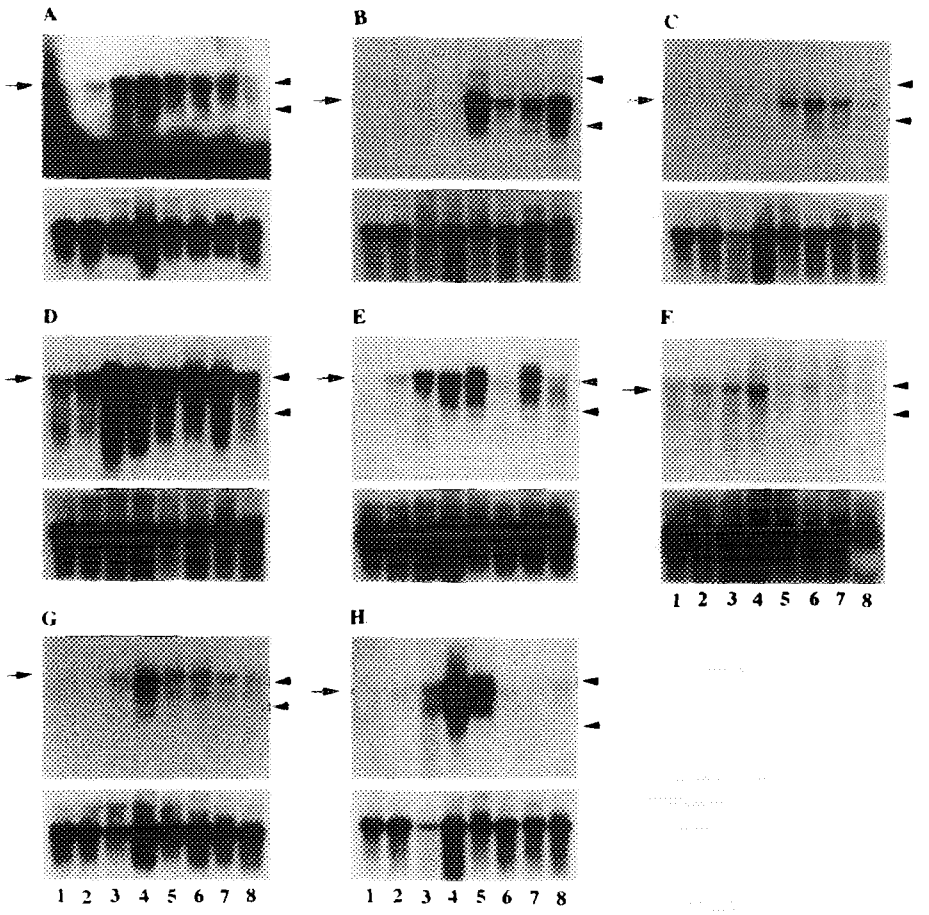

Fig. 2 Expression of non-receptor type PTK genes in liver, kidney, isolated glomeruli, mesangial cells, glomerular endothelial cells, and various renal tubular cell lines: rat liver (lane 1), rat kidney (lane 2), isolated rat glomeruli (lane 3), rat mesangial cells (lane 4), bovine endothelial cells (lane 5), LLCPK1 (lane 6), MDCK (lane 7), and MDBK (lane 8). The blots were hybridized with PTK probes (A, yes; B, lyn; C, tec; D, Jak1; E, Jak2; F, Jak3; G, c-abl; H, flk). The blots were then hybridized with rat EF-1α (lower panel). The sizes of 28S and 18S were indicated on the right.

C). Three tyrosine kinases; hyk, tyro-4, and tie, showed the signals in isolated glomeruli without the significant expression in both mesangial and endothelial cells. Since glomeruli constitute of mesangial, endothelial, and epithelial cells, these kinases may be expressed in glomerular epithelial cells (Fig. 1B, D, and F). Interestingly, tie is known to be exclusively expressed in embryonic neovasculature and endothelial cells (18), the localization of tie transcript in glomeruli should further be addressed to define its specificity in glomeruli. When the gene expressions of tyrosine kinases were compared between *in vivo* (Fig. 1 and 2, lane 1-3) and *in vitro* (Fig. 1 and 2, lane 4-8), four kinases; lyn, tec, c-abl, and flk showed the dominant gene expression in cultured renal cells (Fig. 2B, C, G, and H), arguing that these non-receptor tyrosine kinases may play a role in the cellular proliferation *in vitro*.

Flt-1 is constitutively expressed and up-regulated upon the stimulation of PDGF in mesangial cells.

Flt-1 and Flk-1 are specific receptors for vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), and their expressions were known to be limited to vascular endothelial cells (15,19-21). However, recent reports suggest that Flk-1 is also expressed in beta-cells of pancreatic islet cells (22) and that VEGF induces the differentiation of cultured osteoblasts (23), suggesting the other roles of VEGF system besides vascular angiogenesis. In this communication, we showed for the first time that Flt-1 is constitutively expressed in renal mesangial cells, suggesting that Flt-1 has another biological function in mesangial cells. Interestingly, Flt-1 is not expressed in glomerular endothelial cells (Fig. 3A) while Flk-1 mRNA is detected in isolated glomeruli but hardly detected in cultured endothelial cells (Fig. 3B), suggesting that the expression of VEGF receptors are

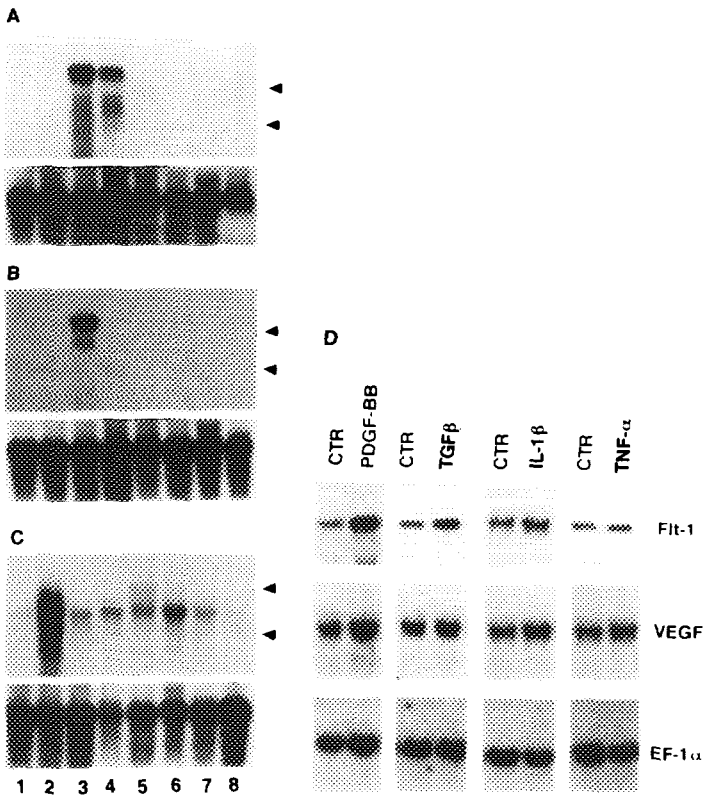

Fig. 3 Expression of VEGF and Flt-1 in renal tissues and in cytokine treated mesangial cells. Flt-1 (A), Flk-1 (B), and VEGF (C) mRNAs were analyzed by Northern blot: liver (lane 1), kidney (lane 2), isolated rat glomeruli (lane 3), rat mesangial cells (lane 4), bovine endothelial cells (lane 5), LLCPK1 (lane 6), MDCK (lane 7), and MDBK (lane 8). D: Cultured mesangial cells were maintained for 48 h in serum-free medium and then treated with 25 ng/ml PDGF-BB, 5 ng/ml TGF-β, 1 x 10^3 U/ml IL-1β, or 1 x 10^3 U/ml TNFα for 12 hrs. Total RNAs were isolated and analyzed by Northern blot. Triplicate filters were hybridized with Flt-1 (upper panel), VEGF (middle panel), and EF-1α (lower panel).

differentially regulated in renal glomeruli. In this context, previous studies suggesting the localization of Flt-1 in glomerular endothelial cells by *in situ* hybridization need to be looked into (15,24). Based on our findings and others (15), it is speculated that the *in situ* signals of Flt-1 may be localized in mesangial cells and those of Flk-1 in endothelial cells. Since VEGF is highly expressed and secreted in renal glomeruli (Fig. 3C; 25-27), it is tempting to speculate that distinct receptor molecules play different roles in mesangial cells and in glomerular endothelial cells for the response to VEGF secreted by glomerular epithelial cells. To analyze further the role of Flt-1 in mesangial cells, we investigated the gene expressions of VEGF and Flt-1 in cultured mesangial cells upon the stimulation of various cytokines known to act on mesangial cells (Fig. 3D). Interestingly, the stimulation of platelet-derived growth factor (PDGF) activate the gene expressions of both Flt-1 and VEGF in mesangial cells while TGF-β, IL-1β, and TNF-α failed to significantly stimulate the gene expressions of Flt-1 and VEGF (Fig. 3D). Since PDGF and IL-1β both induce the potent proliferation of mesangial cells (28,29), the up-regulation of Flt-1 is specific for the mesangial cell proliferation induced by PDGF, but not induced by IL-1β (Fig. 3D). Moreover, in the light that VEGF is also specifically up-regulated in response to PDGF stimulation, this response may be partly based on the autocrine mechanism, although the degree of VEGF up-regulation is less intense (Fig. 3D). In various pathological states, mesangial cells proliferate and change their metabolism of extracellular matrix in response to cytokines, therefore, VEGF/Flt-1 system may be involved in cytokine-induced mesangial cell proliferations. In fact, it is known that PDGF plays a pivotal role in a rat model of mesangial proliferative glomerulonephritis (30,31), the involvement of VEGF/Flt-1 system in these mesangial proliferative responses *in vivo* should be addressed in the subsequent study.

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