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# Intraglomerular expressions of IL-1α and platelet-derived growth factor (PDGF-B) mRNA in experimental immune complex-mediated glomerulonephritis

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## SUMMARY

Both PDGF and IL-1 play important roles as autocrine growth factors for cultured mesangial cells, and may be closely associated with the progression of glomerulonephritis. In this study we investigated intraglomerular expressions of PDGF-B and IL-1α mRNA in mice with bovine serum albumin (BSA) nephritis, a model of immune complex-mediated glomerulonephritis, using the reverse transcription-polymerase chain reaction (RT-PCR) method. We also quantified intraglomerular PDGF-B mRNA by the competitive PCR and studied the correlation between the level of intraglomerular PDGF-B mRNA expression and the degree of observed glomerular injury. While expression of neither PDGF-B nor IL-1α mRNA was detected in glomeruli from control mice, both were strongly expressed in glomeruli from mice with BSA nephritis. IL-1α mRNA in glomeruli showed low accumulation in mice with mild glomerular injury, and was increased in mice with moderate glomerular injury. In contrast, high intraglomerular expression of PDGF-B mRNA occurred in all mice with mild glomerular injury and continued throughout the course of the disease. We observed no correlation between the level of PDGF-B mRNA expression and the histologic grade of renal damage. These results suggest that PDGF and IL-1 have different growth properties, and PDGF might play a role as a competence factor rather than a progression factor in the pathogenesis of immune complex-mediated glomerulonephritis.

**Keywords** IL-1 glomerulonephritis competence factor progression factor platelet-derived growth factor

## INTRODUCTION

PDGF is a 30-kD protein dimer composed of subunits encoded by separate genes (PDGF-A and PDGF-B). PDGF exists in three different forms, as a heterodimer (PDGF-AB) and as two homodimers (PDGF-AA and PDGF-BB) [1]. In platelets, PDGF is stored in α granules and is released following platelet activation by a variety of substances. PDGF is also synthesized and released by various other cell types [1], and it has mitogenic effects on cells of mesenchymal origin, including glomerular mesangial cells [2–13]. Studies of cultured mesangial cells demonstrated that these cells proliferated in response to PDGF, released PDGF and expressed PDGF mRNA [14,15].

*In vitro* studies also have demonstrated that PDGF acted as a competence factor for cultured mesangial cells rather than as a progression factor, suggesting that PDGF may induce mesangial cells to enter the cell cycle, while other factors, such as IL-1, may promote mesangial cell growth as progression factors [16]. Moreover, recent studies have demonstrated intraglomerular expression of PDGF or PDGF receptor mRNA in experimental glomerulonephritis using Northern blotting or *in situ* hybridization [17–19]. It has also been reported that mesangial cell proliferation could be reduced by administering a neutralizing anti-PDGF IgG [20]. These *in vivo* studies showed both increased expression of PDGF mRNA in experimental glomerulonephritis and the importance of PDGF in the pathophysiology of glomerulonephritis. However, because Northern blotting or *in situ* hybridization are insensitive and not quantitative, the correlation between the levels of intraglomerular mRNA expression and renal histopathology in a single mouse could not be investigated.

To overcome these problems, we introduced the reverse transcription-polymerase chain reaction (RT-PCR) technique. RT-PCR is so sensitive that we can investigate the levels of intraglomerular mRNA expression for PDGF-B and IL-1α in a single mouse and can clarify the role of PDGF-B and IL-1α in

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the pathogenesis of experimental immune complex-mediated glomerulonephritis. Furthermore, we measured the amount of intraglomerular PDGF-B mRNA using the competitive PCR method established by Gilliand *et al.* [21] in order to clarify the role of PDGF-B in this experimental glomerulonephritis.

## MATERIALS AND METHODS

*Induction of chronic serum sickness nephritis*

Female C57Bl/B10-BR mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Chronic serum sickness nephritis was induced by a method previously described [22]. Eighty mice were preimmunized for 8 weeks by s.c. injection of 0·2 mg/mouse bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO) in Freund's incomplete adjuvant (FIA) at 2-week intervals. After this preimmunization we divided the mice into two groups: the first received daily i.p. injections of 50 mg/kg BSA for 1 week and were then killed. This group of mice were killed at that time point in order to produce the mild renal injury. The second group received i.p. injections of 50 mg/kg BSA daily for 4 weeks. This group of mice showed moderate or severe renal injury. We chose these two time points in order to produce different degrees of renal involvement.

As controls, 20 C57Bl/B10-BR mice were preimmunized with BSA and then injected daily with PBS instead of BSA.

*Kidney samples*

After sacrifice, each mouse's kidneys were removed. Specimens of the kidneys were fixed in 10% formalin and embedded in paraffin for the microscopic examination. Glomeruli were isolated from the rest.

*Histologic studies of kidney sections*

After staining with periodic acid-Shiff, kidney sections were examined light microscopically, and the severity of renal involvement was classified as follows:

*Mild*: a mild increase in mesangial cell number and mesangial matrix was evident in almost all glomeruli. Kidney sections from all mice, which were given daily i.p. BSA injections for 1 week, were classified as mild.
*Moderate*: a moderate increase in mesangial cell number and widening of the mesangial matrix were present in almost all glomeruli. Crescent formation was occasionally observed.
*Severe*: marked increases in mesangial cell number and expansions of mesangial matrix were present in almost all glomeruli. Many glomeruli had cellular crescents.

Figure 1 presents representative light micrographs of these different severities of renal involvement of BSA nephritis. Classification was performed by two experts in renal pathology blind to the purpose of this study.

While all mice ($n = 15$) killed following daily BSA injections for 1 week showed mild renal involvement, among mice killed after daily BSA injections for 4 weeks 22 mice showed moderate renal involvement and 23 severe renal involvement. Among mice with mild renal involvement ($n = 15$), eight mice were used for RT-PCR and seven were used for the competitive PCR. Among 22 mice with moderate renal involvement 10 mice were used for RT-PCR and another 10 for the competitive PCR, and among mice with severe renal involvement 11 mice for RT-PCR and 10 for the competitive PCR.

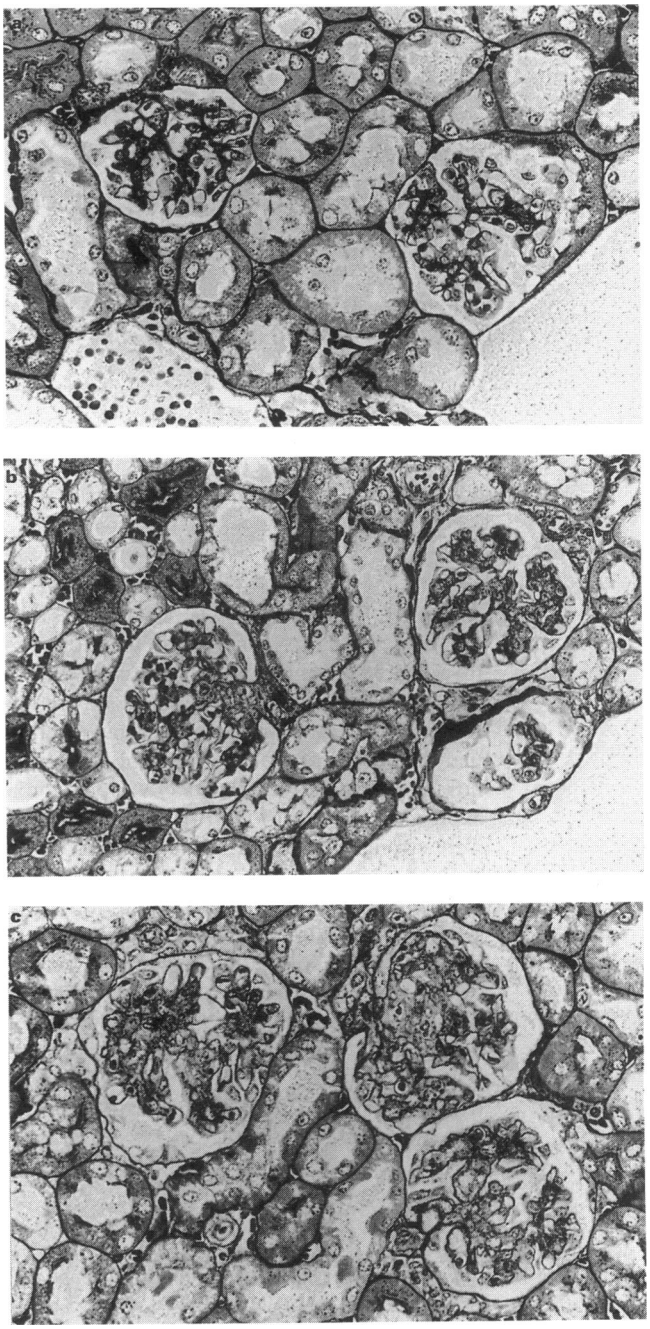

**Fig. 1.** Light micrographs of kidney sections obtained from bovine serum albumin (BSA) nephritis mice. (a) Mild. The glomeruli show mild increases of mesangial matrix and mesangial cell numbers (periodic acid-silver methenamine (PAM) staining, ×340). (b) Moderate. The glomeruli show moderate mesangial proliferation (PAM staining, ×340). (c) Severe. The mesangial areas are markedly enlarged and show a tendency to lobulation (PAM staining, ×340).

*Isolation of glomeruli*

Instead of the sieving method, we used a new and more efficient method developed in our laboratory [23]. In brief, renal cortices

**Table 1.** Primer sequences

| | Upper | Lower | Product size (bp) |
|---|---|---|---|
| PDGF-B | 5′-CTGTATGAAATGCTGAGCGACCA-3′ | 5′-GCATTGCACATTGCGGTTATTGC-3′ | 343 |
| IL-1α | 5′-CTCTAGAGCACCATGCTACAGAC-3′ | 5′-TGGAATCCAGGGGAAACACTG-3′ | 288 |
| β-actin | 5′-GTGGGCCGCTCTAGGCACCA-3′ | 5′-TGGCCTTAGGGTGCAGGGGG-3′ | 240 |

were minced into small pieces followed by a treatment with collagenase type IV (Worthington Biochemical Co., Freehold, NJ). Renal cortices were then centrifuged over a discontinuous 30%/50% Percoll gradient (Pharmacia Inc., Piscataway, NJ), and the 30%/50% interface was collected. Gradient centrifugation was repeated to yield highly purified glomeruli.

*Preparation of RNA*

Total RNA was isolated from glomeruli using the guanidinium thiocyanate procedure [24]. Two micrograms of RNA were then size fractionated by electrophoresis on a 1% agarose gel and visualized with ultraviolet illumination to assess the integrity of the RNA and to verify its concentration.

*Synthesis of cDNA*

Total RNA (0·5 μg) was converted to cDNA by reverse transcription using 1 U of reverse transcriptase RAV 2 (Amersham, Aylesbury, UK) and the following reagents (in a total volume of 20 μl): 10-fold reaction buffer (10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% gelatin) (Perkin Elmer Cetus, Norwalk, CT), 20 U of human placental RNase inhibitor, random hexanucleotides (15 μg/ml), 1 mM dATP, 1 mM dGTP, 1 mM dTTP and 0·5 mM dCTP (Amersham). The reaction was carried out at 42°C for 60 min and was stopped by heating the solution at 95°C for 5 min. The samples were then stored at −20°C until use.

*Oligonucleotide primers*

The primers used in this study are shown in Table 1. Primers for PDGF-B were designed using the published sequence of genomic PDGF-B DNA [25]. Published sequences were also used for IL-1α [26] and β-actin [27] primers. Primers of upper and lower strands were designed to prime different exons, and were synthesized by the phosphoamidite solid-phase method on a PCR-MATE 391 DNA synthesizer (Applied Biosystems, Foster, CA) and purified using Oligonucleotide Purification Cartridges (Applied Biosystems).

*Polymerase chain reaction*

PCR was performed using the GeneAmp PCR Reagent Kit (Perkin Elmer Cetus). Reactions were performed in a total volume of 100 μl consisting of 1 μl of the cDNA preparation, 10-fold reaction buffer (10 mM Tris-HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·001% gelatin), dNTPs (10 μM each), 2·5 U of Taq DNA polymerase and 0·2 μM of each primer. A Thermal Cycler (Perkin Elmer Cetus) was used for amplification. The PCR was carried out for 40 cycles. The cycle conditions for PDGF-B were denaturation at 95°C for 1 min, annealing at 58°C for 30 s and extension at 72°C for 1 min. The conditions for amplification of IL-1α were similar to those of PDGF-B except for annealing at 67°C. β-actin was used as an internal control. Reaction products were analysed by electrophoresis on 2% Nu Sieve 3:1 agarose gels (FMC BioProducts, Rockland, ME).

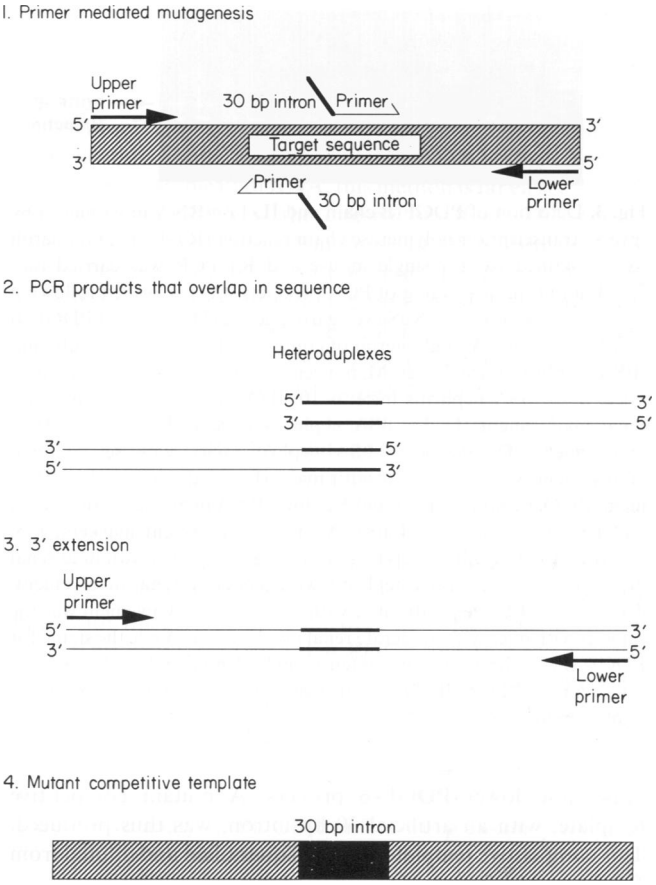


**Fig. 2.** Scheme for the generation of the mutant competitive template with an artificial intron.

*Generation of competitive template*

Competitive template, with the insertion of 30 extra bp, was generated as follows. As shown in Fig. 2, we made internal primers with added, complementary, 5′ sequences of 30 bp. The sequences of upper and lower internal primers were 5′ - ATGAGCCAGCACGTCTAACTATGCTTTCCCTCG-AGGGAGGAGGAGCCTAGG-3′ and 5′-GGGAAAGCAT-AGTTAGACGTGCTGGCTCATGATGAGCTTTCCAAC-TCGACT-3′, respectively. PCR was carried out using each of these primers and one of the original upper or lower PDGF-B primers. Two PCR products that overlapped for 30 bp at their 5′ ends were thus generated. By denaturing and reannealing these PCR products, heteroduplexes were generated. Following 3′ extension, these products were reamplified using the original

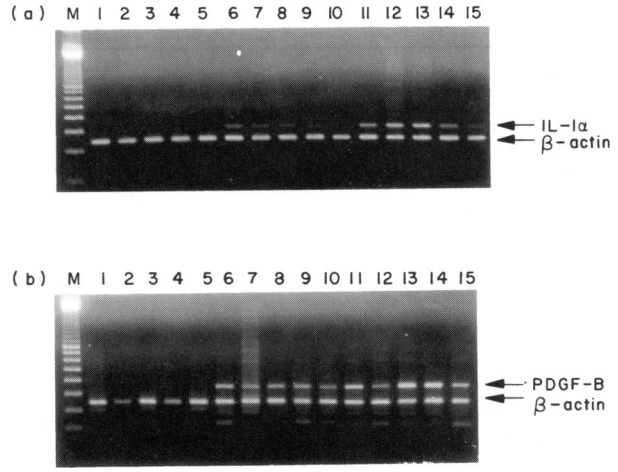


**Fig. 3.** Detection of PDGF-B chain and IL-1α mRNA in glomeruli by reverse transcription-polymerase chain reaction (RT-PCR). Glomeruli were obtained from a single mouse and RT-PCR was carried out. Ethidium bromide staining of PCR products was performed following electrophoresis on a 2% NuSieve agarose gel. (a) Detection of PDGF-B and β-actin mRNA in glomeruli of control and bovine serum albumin (BSA) nephritis mice. Lanes: M, molecular weight markers; 1–5, control mice; 6–15, BSA nephritis mice: 6–10, BSA nephritis mice with mild renal involvement, 11–15, BSA nephritis mice with moderate renal involvement. The result of BSA nephritis mice with severe renal involvement was the same as with mice with moderate renal involvement. (b) Detection of IL-1α and β-actin mRNA in glomeruli of control and BSA nephritis mice. Lanes: M, molecular weight markers; 1–5, control mice; 6–15, BSA nephritis: 6–10, BSA nephritis with mild renal involvement, 11–15, BSA nephritis with moderate renal involvement. The result of BSA nephritis mice with severe renal involvement was the same as with mice with moderate renal involvement. While the signal for PDGF-B was almost the same intensity in both mild and moderate renal involvement, that for IL-1α was stronger in moderate renal involvement than in mild.

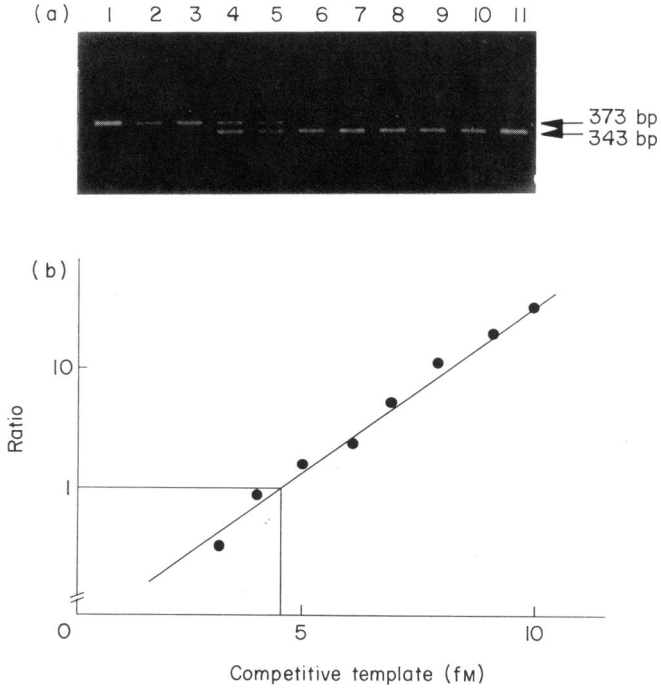


**Fig. 4.** Quantitative analysis of PDGF-B chain mRNA in bovine serum albumin (BSA) nephritis glomeruli from a single mouse. (a) Competitive polymerase chain reaction (PCR) was performed in a reaction mixture containing authentic and competitive mutant PDGF-B cDNA. Lanes: 1–11, the starting concentrations of competitive mutant template in each tube (left to right) were 50, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 fM, respectively. Bands corresponding to the PCR products of both PDGF-B cDNA (343 bp) and competitive template (373 bp) were observed. Results were analysed by densitometry. (b) Densitometry data, plotted as the ratio of authentic PDGF-B to mutant competitive template *versus* the known input concentration of competitive template, were linear on a linear-log scale. To correct for the difference in the sizes of these products, the raw density of the competitive signal was multiplied by 343/373.

upper and lower PDGF-B primers. A mutant competitive template, with an artificial 30 bp intron, was thus produced. This competitive template was then quantified and diluted from 100 fM to 0·01 fM.

*Competitive PCR*

Quantification of PDGF-B mRNA was done using the competitive PCR method described previously [20]. The procedure was similar to the PCR used above, except for the addition of competitive template to the reaction mixture. The cycle conditions of amplification and the amplification number were the same as those for PDGF-B described above. The reaction products were subjected to densitometry after electrophoresis on a 2% Nu Sieve agarose gel and staining with ethidium bromide.

## RESULTS

*Expressions of PDGF-B mRNA and IL-1α mRNA in glomeruli*

We investigated intraglomerular mRNA expressions of PDGF-B and IL-1α using RT-PCR and compared these mRNA expressions with the severity of renal involvement. We amplified intraglomerular β-actin as an internal control. IL-1α mRNA expression was absent from controls and only weakly detected in mice with mild renal involvement. Expression of IL-1α mRNA was increased in mice with moderate and severe renal involvement. In contrast, PDGF-B mRNA expression was elevated even in mice with mild renal involvement, and a high level of expression was also observed in mice with moderate and severe renal involvement. The β-actin expression remained at about the same level in all glomeruli (Fig. 3).

*Quantification of PDGF-B mRNA in glomeruli*

RT-PCR analysis of PDGF-B mRNA suggests that PDGF-B expression is approximately constant throughout the course of BSA nephritis. Since RT-PCR was suitable for the detection of small amounts of mRNA but not for its accurate quantification, we used competitive PCR in order to confirm the RT-PCR result. As shown in Fig. 4a, samples containing large amounts of the PDGF-B mutant competitive template generated almost exclusively a 373 bp product. As the amount of competitive template added was reduced, the intensity of this band decreased, and a 343 bp product, expected to derive from authentic PDGF-B mRNA, gained in intensity. Densitometry data, plotted as the ratio of authentic PDGF-B to mutant competitive signals *versus* the known concentration of input competitive template, were linear when on a linear-log scale (Fig. 4b). At the point where authentic PDGF-B and mutant product signals were

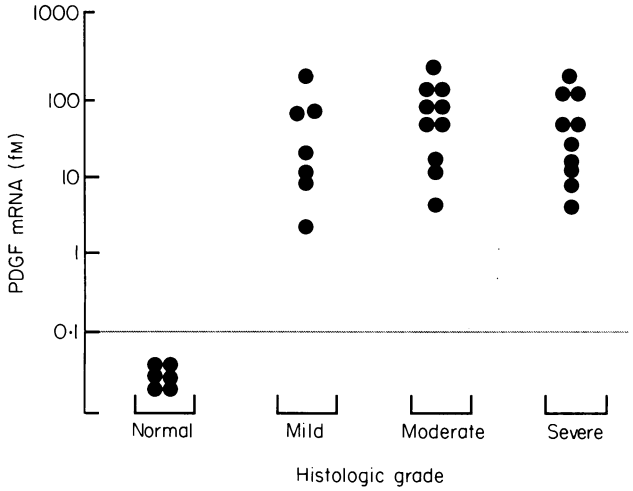


**Fig. 5.** Correlation between the amount of PDGF-B mRNA and the severity of renal histopathology. Whereas intraglomerular expression of PDGF-B mRNA was not detected in control glomeruli, a high level of PDGF-B mRNA expression was observed from the stage of mild renal involvement and was maintained in the stages of moderate and severe renal involvement. Significant correlation was not observed between the amount of intraglomerular PDGF-B mRNA expression and the grade of renal histopathology.

equivalent (ratio = 1), the initial concentration of PDGF-B mRNA was equal to the starting concentration of competitive template. This method allowed us to quantify any PDGF-B mRNA expression greater than 100 aM. Whereas the amount of PDGF-B mRNA expression in control glomeruli was too small to be quantified, the expression ranged from 3 fM to 100 fM in BSA nephritis mice, i.e. at least 30 times that of controls (Fig. 5).

*Correlations between the amount of PDGF mRNA expression and renal histopathology*

To clarify the role of PDGF in BSA nephritis, we examined intraglomerular expression of PDGF mRNA during the progress of renal pathology. High levels of PDGF-B mRNA expression were detected even in mice with mild renal injury. Although this high expression continued to end-stage renal pathology, the amount of PDGF mRNA in glomeruli did not correlate with the degree of renal histopathology (Fig. 5).

## DISCUSSION

A recent report by Iida *et al.* has reported that PDGF mRNA could be detected in glomeruli obtained from three to six rats with anti-Thy 1 glomerulonephritis [28]. Gesualdo *et al.* have reported that, in IgA nephropathy mice, the expression of PDGF-B chain mRNA was detected in whole kidney samples, not in isolated glomeruli [18]. These studies did not show the expression of mRNA encoding for PDGF-B in a homogeneous experimental preparation, because conventional methods such as Northern blotting required larger amounts of mRNA. In this study, the RT-PCR method was used to detect intraglomerular expressions of mRNAs. This method enabled us to detect small amounts of mRNA expressed in glomeruli of an individual BSA nephritis mouse, and thereby evaluate the relation between mRNA expression and renal histology in an individual BSA nephritis mouse.

Moreover, we used the competitive PCR method for the quantification of PDGF-B mRNA in glomeruli of an individual diseased mouse. Quantification of mRNA expression is important because protein level, and thus activity, are often regulated by quantitative changes in mRNA levels. Conventional methods of mRNA detection, such as Northern blotting and *in situ* hybridization which have relatively low sensitivity, are therefore inadequate for quantitative analysis. Several more sensitive methods of quantifying mRNA have been developed recently. Competitive PCR [20] uses synthetic mutant cDNA, which has the same sequence as the target except for the addition of a restriction site by a single base substitution or the insertion of a short intron as the internal control. Since the sequence of the competitor is almost identical to that of the target, differences of amplification efficacy should be negligible. Furthermore, as DNA is more stable than RNA, this method is far easier than the method using cRNA as the internal standard. Therefore, it allowed us to quantify small amounts of mRNA precisely and easily. However, since we can not know the efficacy of reverse transcription, competitive PCR method used in this study can give relative, rather than absolute, quantification of mRNA, i.e. it is semi-quantitative. The PCR-aided transcript titration assay (PATTY) [29] uses synthetic mutant cRNA as the internal standard and can give absolute quantification of the target mRNA. However, RNA is labile and easily digested by RNases which often contaminate experimental specimens and instruments, so that precise quantification of mRNA by this method requires much effort.

This study demonstrated that while intraglomerular expressions of PDGF-B chain and IL-1α mRNA were increased in mice with BSA nephritis, both were not detectable in control mice. This result suggested that PDGF and IL-1 were closely associated with the pathogenesis of BSA nephritis, consistent with earlier reports of PDGF-B chain mRNA expression in experimental glomerulonephritis [16,17,28]. PDGF-B mRNA expression was detected in mice with mild renal involvement and was sustained throughout the disease course. Moreover, in the quantitative analysis, PDGF-B mRNA expression showed no correlation with the degree of renal injury. In contrast, the expression of IL-1α mRNA was weakly detected in mice with mild renal involvement and was increased in mice with moderate renal histology. These results suggested that PDGF acted differently from IL-1 in the pathogenesis of immune complex-mediated glomerulonephritis. However, since we cannot identify the cells producing IL-1α and PDGF in this study, IL-1α mRNA expression might be partly due to infiltrated inflammatory cells.

Shultz *et al.* have indicated that PDGF was the complete growth factor for cultured mesangial cells by the facts that PDGF bound to specific receptors on mesangial cells and stimulated DNA synthesis and cellular proliferation [14]. However, some reports suggested that PDGF played a role in inducing cells to enter the cell cycle as a competence factor for cultured mesangial cells, as is the case in other eukaryotic cells [30,31]. Lovett *et al.* indicated that rat mesangial cells proliferated in response to IL-1, when they were cultured in 20% serum, in which PDGF was the principal mitogen present, but not when they were cultured in PDGF-depleted serum. They thus suggested that mesangial cells were rendered competent by PDGF and were then capable of responding to added IL-1 [16]. Recently, Doi *et al.* reported that PDGF enhanced the action of

insulin-like growth factor-1 (IGF-1) for the proliferation of cultured human mesangial cells and that PDGF and IGF-1 acted at different points of the cell cycle [32]. PDGF would thus make mesangial cells competent for the action of other growth factors, including IL-1 and IGF-1, which allow cells to complete the cell cycle as progression factors. In this study we showed the difference in the manner of intraglomerular mRNA expressions of PDGF and IL-1, and these results were consistent with the *in vitro* results described above. Therefore, we suggest that PDGF might have served as a competence factor, while other factors, perhaps including IL-1, affected the growth properties of glomerular cells in immune complex-mediated glomerulonephritis. However, to clarify the more precise mechanism of these growth factors *in vivo*, further studies are required on the kinetics of these factors.

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