incidence as follows: 0–4%, 5–24%, 25–49%, 50–74%, and 75–100%.

Measurement of serum TGF-β1 concentration

We could examine serum sample at the time of biopsy, before any specific treatment performed, from only a portion of the patients studied. In 78 patients with IgAN, whose serum samples at the time of diagnosis were available, serum levels of TGF-β1 were measured by an enzyme-linked immunosorbent assay (ELISA) kit (Amersham Bioscience, Pisctaway, NJ). According to the manufacturer's instruction manual, the detection limit of this assay was 4 pg/ml, and the intra-assay and interassay coefficients of variance were ≤3.9 and ≤13.4%, respectively. The assay showed essentially no cross-reactivity (≤1%) with TGF β2, TGF β3, or other cytokines.

Genotype determination

Genomic DNA of peripheral blood cells was isolated by an automatic DNA isolation system (NA-1000; Kurabo, Osaka, Japan). The genotypes of *C-509T* and *T869C* of *TGF β1* gene were determined by polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) as described previously (17, 21). PCR primers for the *C-509T* polymorphism were 5′-GGGGACACCATCTACAGTG-3′ (forward) and 5′-GGAGGAGGGGGCAA CAGG-3′ (reverse), and those for *T869C* were 5′-TTCAAGACCACCCACCT TCT-3′ (forward) and 5′-TCGCGGGTGCTGTTGT ACA-3′ (reverse), respectively. The reaction mixture contained 1 × PCR buffer, 1.5 mmol/l $MgCl_2$, 200 mmol/l deoxynucleotide triphosphates (dNTPs), 1unit Taq DNA polymerase (Takara, Kyoto, Japan), 10 pmol of each primer, and 50–100 ng of genomic DNA. The PCR amplification reaction consisted of a cycle at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s (*C-509T*) or at 60°C for 30 s (*T869C*), and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. The PCR products were digested with restriction endonuclease *Eco*81I (MBI Fermentas, Hanover, MD) for *C-509T* and *Msp*AlI (New England Biolabs Inc., Beverly, MA) for *T869C*, respectively, and electrophoresed on a polyacrylamide gel. The gel was silver stained with using the DNA Silver Staining Kit (Pharmacia Biotech, Pisctaway, NJ). For *C-509T* polymorphism, the T alleles resulted in a 430-bp and 25-bp fragment, whereas the 455-bp – *509C* alleles lacked its restrictive site (Fig. 1A). For *T*869C polymorphism, 273-bp and 12-bp fragment were detected for 869C alleles, whereas 285-bp *869T* alleles lacked its restrictive site, and the complete digestion was confirmed by disappearance of the 500-bp band, which corresponded to the PCR product without *MSP*AlI digestion (Fig. 1B).

Haplotype estimation and statistical analysis

Haplotype frequencies for sets of alleles were estimated using ARLEQUIN software Ver 2.0, which was based on the maximum likelihood method (Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland; http://www.lgb.unige.ch/arlequin/). Pairwise linkage disequilibrium coefficients (D′) were also

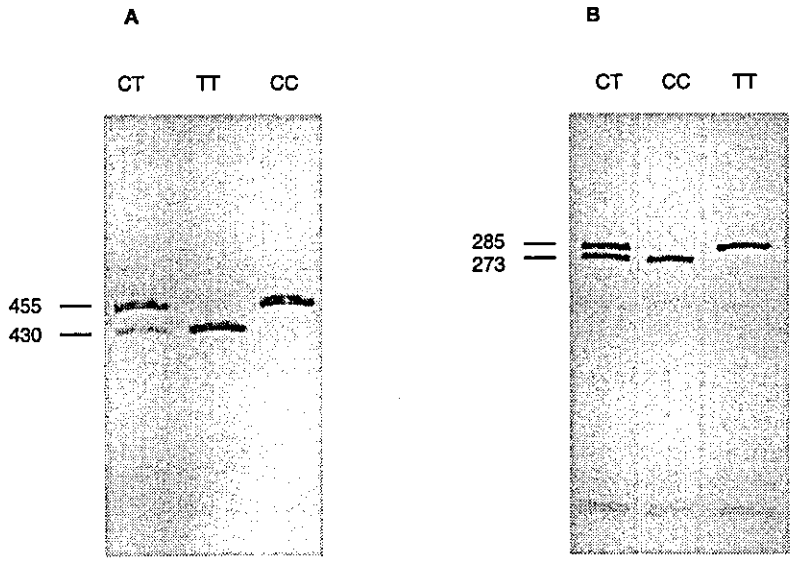

Fig. 1. **Polyacrylamide gel elecrophoresis of PCR products after restriction digestion.** Each genotype of *C-509T* (A) and *T869C* (B) was clearly defined by the method described.

calculated using ARLEQUIN Ver 2.0 and expressed as the $D' = D/Dmax$, according to Slatkin (22).

STATVIEW 5.0 statistical software (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analyzes. Hardy–Weinberg equilibrium was tested by a χ^2-test with 1 d.f. Clinical data were compared between different genotypes of both *-509* and *869* positions by Kruskal–Wallis test or Mann–Whitney *U*-test. Values of $P < 0.05$ were considered to indicate statistical significance. When it was significant by Kruskal–Wallis test, we performed the Mann–Whitney *U*-test to compare each pair of genotype groups, adjusting by Bonferoni correction, where $P < 0.0167$ was considered statistically significant.

Results

In total, we genotyped 626 subjects, which consisted of 329 patients with histologically proven IgAN and 297 healthy controls. Table 1 summarizes the genotype distributions, allele frequencies, and estimated haplotype frequencies of *C-509T* and *T869C* polymorphisms of *TGF-β1* gene in patients with IgAN and normal controls. The genotype distributions were similar between the patients with IgAN and controls, and the allele frequencies were in accordance with previous reports in a Japanese population (19). The expected frequencies of the genotypes, assumed to be under Hardy–Weinberg equilibrium, were no different from the observed genotype distributions in both the patients with IgAN and the controls (data not shown). We found that the *C-509T* and *T869C* polymorphisms were in tight linkage disequilibrium ($D' = 0.9401$; $P < 0.0001$) and that the major haplotypes for these two loci were *C-C* and *T-T*, which accounted for more than 95% of the total chromosomes. The frequencies of these two major haplotypes were similar between patients with IgAN and healthy controls.

Genotype distributions, allele frequencies, and estimated haplotype frequencies of *C-509T* and *T869C* polymorphisms of *TGF-β1* gene in patients with IgAN and normal controls

			IgAN ($n=329$)	Control ($n=297$)	*P*-value	χ^2
C-509T	Genotype	*CC*	89 (0.271)	76 (0.256)		
		CT	174 (0.529)	157 (0.529)		
		TT	66 (0.201)	64 (0.215)	0.8637	0.293
	Allele	*C*	0.535	0.520		
		T	0.465	0.480	0.6809	0.169
T869C	Genotype	*CC*	86 (0.261)	80 (0.269)		
		CT	167 (0.508)	152 (0.512)		
		TT	76 (0.231)	65 (0.219)	0.9301	0.145
	Allele	*C*	0.515	0.525		
		T	0.485	0.475	0.7722	0.084
Estimated haplotype of *C-509T* and *T869C*						
	-509	869				
	C	*C*	0.501	0.515	0.6300	0.232
	T	*T*	0.451	0.470	0.5158	0.422
	Others		0.048	0.015		

IgAN, IgA nephropathy.

Table 1

Table 2 summarizes the demographic data and clinical characteristics at the time of diagnosis among IgAN patients with each genotype of the *C-509T* and *T869C* polymorphisms. There was no significant difference among patients with each genotype of both polymorphisms in gender, age, height, weight, and time from the first urine abnormality to renal biopsy, serum creatinine, creatinine clearance, serum IgA, or blood pressures. However, in patients with *CC* genotype of *C-509T* polymorphism and in those with the *CC* of *T869C*, urinary protein excretion was significantly higher than in those with other genotypes (Kruskal–Wallis test, $P = 0.0051$ for *C-509T*, and $P = 0.0241$ for *T869C*). Even after Bonferoni correction in the Mann–Whitney *U*-test, patients with the *-509CC* and *869CC* genotypes had significantly higher urinary protein excretion than those with *-509CT* ($P = 0.0011$) and *869CT* ($P = 0.0038$), respectively. Although the differences were no longer significant after Bonferoni correction between *-509CC vs -509TT* ($P = 0.0178$) and *869CC vs 869TT* ($P = 0.0853$), we further examined the association between these polymorphisms in the incidence of proteinuria of 1.0 g/day or more. The percentage of patients with urinary protein excretion of 1.0 g/day or more was significantly higher in patients with the *-509CC* ($\chi^2 = 11.003$, $P = 0.0041$) and those with the *869CC* of *TGF-β1* polymorphism ($\chi^2 = 9.382$, $P = 0.0092$) than in those with other genotypes. Table 3 summarizes estimated haplotype frequencies in patients with or without urinary protein excretion of 1.0 g/day or more. The *C-C* haplotype of these polymorphisms was significantly more frequent in patients with urinary protein excretion of 1.0 g/day or more than in those with less proteinuria ($\chi^2 = 11.782$, $P = 0.0006$).

Next, we investigated possible association between the *TGF-β1* polymorphisms and histopathological findings of kidney biopsy including glomerular and interstitial changes. Figures 1 and 2 show the mean scores for mesangial cell proliferation, mesangial matrix increase, endocapillary proliferation, duplication of GBM, crescent formation, adhesion of tufts to Bowman's capsule, and tubulointerstitial lesions in patients with each genotype of *TGF-β1*. Glomerular cell proliferation was significantly higher in patients with the *-509CC* (Fig. 1, Mann–Whitney *U*-test, $P = 0.0054$) and with the *869CC* genotype (Fig 2, $P = 0.0052$) than that in those with other genotypes, whereas no difference was detected in terms of any other histopathological scores (data not shown). Moreover, the

Demographic data and clinical characteristics at time of diagnosis among patients with each genotype of the C-509T and T869C polymorphisms

		C-509T				*T869C*			
	Total ($n=329$)	CC ($n=89$)	CT ($n=174$)	TT ($n=66$)	*P*-value	CC ($n=86$)	CT ($n=167$)	TT ($n=76$)	*P*-value
Gender (male/female)	149/180	48/41	75/99	26/40	0.1675	41/45	77/90	31/45	0.8950
Age (years)	36.9±13.8	36.8±13.3	37.1±13.8	37.3±14.8	0.9958	36.8±12.9	37.5±14.1	36.5±14.3	0.8682
Height (cm)	161.1±9.5	160.5±10.9	161.4±8.9	161.4±8.4	0.9845	160.7±9.6	161.9±8.7	160.0±10.5	0.4418
Weight (kg)	58.8±9.7	59.7±10.2	58.5±9.7	58.3±9.7	0.3924	59.8±9.3	58.7±9.8	57.9±10.5	0.5103
Urinary protein (UP) excretion (g/day)	1.39±1.52	1.74±1.67	1.27±1.54	1.27±0.17	0.0051	1.67±1.67	1.25±1.54	1.39±1.42	0.0241
Patients with UP of 1.0 g/day or more (%)	48.3	62.8	41.3	47.5	0.0041	60.5	41.6	49.3	0.0092
Serum creatinine (mg/dl)	0.98±0.59	0.97±0.44	1.02±0.63	0.94±0.70	0.2125	0.95±0.44	1.02±0.63	0.96±0.69	0.3180
Creatinine clearance (ml/min)	88.8±32.6	91.1±31.6	85.0±31.7	95.5±35.0	0.1293	91.9±32.0	85.4±31.0	92.6±35.6	0.2677
Systolic blood pressure (mmHg)	128.3±18.8	129.5±21.2	127.9±18.2	126.7±16.4	0.8989	129.3±21.5	127.7±18.3	127.4±16.2	0.9948
Diastolic blood pressure (mmHg)	77.4±13.7	77.3±13.0	77.9±13.8	75.9±13.6	0.6679	76.9±13.6	77.6±13.7	77.0±13.4	0.8799
Serum IgA concentration (mg/dl)	355.6±115.8	375.5±138.7	349.7±105.7	360.6±122.1	0.5447	374.6±135.5	350±108.7	359.2±121.0	0.4978
Time from the first urine abnormality to renal biopsy (years)	5.2±6.3	5.6±7.2	4.9±6.1	5.2±6.1	0.6029	5.7±7.2	5.0±6.1	4.8±6.0	0.5228

Table 2

prevalence of cases with cell proliferation of grade 2 (diffuse mild) or more was significantly higher in patients with the *-509CC* ($\chi^2=7.095$, $P=0.0077$) and with *869CC* genotype of *TGF-β1* ($\chi^2=5.651$, $P=0.0172$) than in those with other genotypes.

Because several investigators have already reported associations between TGF-β1 gene polymorphisms and the circulating level of TGF-β1 in non-nephritic populations (6, 7, 18, 19), the circulating level of TGF-β1 in patients with IgAN was investigated in this study

Estimated haplotype frequencies in patients with or without urinary protein excretion of 1.0 g/day or more

	Urinary protein of 1.0 g/day or more			
Estimated haplotype of −509 and 869 loci	Yes ($n=318$)	No ($n=390$)	χ^2	*P*-value
C-C	181	148	11.78	0.0006
T-T	123	175	10.85	0.0010
Others	14	17	–	–

Table 3

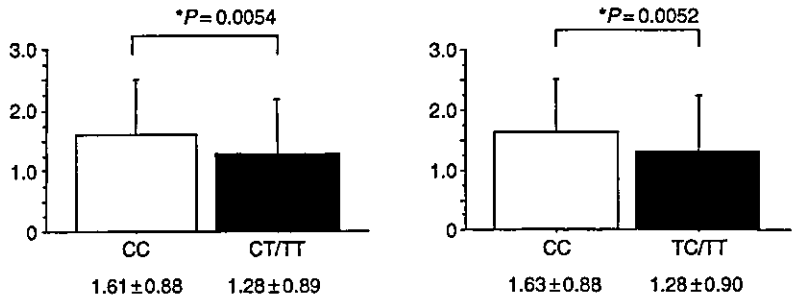

Fig. 2. **Mean values of mesangial cell proliferation in IgA nephropathy (IgAN) patients with the *CC* (□) and *TC/TT* (■) genotypes of the *TGF-β1 C-509T* (A) *and T869C* (B) polymorphisms.** Glomerular changes were scored for each glomerulus, and the mean score of each was calculated. The scores for mesangial cell proliferation were graded from zero to four as described in Method. Data are given as mean ± SD. $^*P<0.05$ by Mann–Whitney *U*-test.

by ELISA. In only 78 patients, the serums at the time of biopsy were available before any specific treatment. There was no significant difference in serum TGF-β1 concentrations among each genotype of *C-509T* and *T869C* (data not shown).

Discussion

TGF-β1 is an attractive and promising candidate for genetic studies of glomerular diseases, because this cytokine has been well documented to be one of the key mediators contributing to the initiation and progression of renal injury and the production of TGF-β1 has been reported to be under genetic control (6, 7). Moreover, this cytokine acts on B-lymphocytes in IgA class switching. Therefore, this study examined possible associations between the genetic polymorphisms of *TGF-β1* and the development of IgAN, as well as the clinical and histopathological manifestations of patients with histologically proven IgAN. There was no difference in genotype, allele frequencies, or estimated major haplotype frequencies of these polymorphisms between IgAN patients and healthy controls, indicating that the *TGF-β1* polymorphisms investigated in the present study have no major involvement in the initiation of IgAN, while they were associated with heavy proteinuria and mesangial cell proliferation within patients with IgAN. This may support the notion that the impact of polymorphisms on phenotype depends on the specific disease under study.

Both polymorphisms investigated in this study, *C-509T* and *T869C* in the *TGF-β1* gene, have been reported to be associated with the transcriptional activity of the gene or the serum level of the gene product (7, 19). Although *G915C* polymorphism, which results in the change of codon 25 from arginine to proline, is another gene variation in the first exon of *TGF-β1* and is the best evaluated in Caucasian populations, it has been known that there is no C allele of this polymorphism in Japanese (13, 14).

The haplotype analysis revealed that *C-509T* and *T869C* loci are in tight linkage disequilibrium, and the major haplotypes were *C-C* and *T-T*. Although the present study could not confirm or completely refuse the possible association between these polymorphisms and circulating level of TGF-β1, our result is consistent with a report in a Japanese population (17). It would be more valuable to assess the local expression of TGF-β1 in the kidney, because TGF-β1 acts as an auto- or paracrine factor in local tissue injury rather than as a systemic circulating factor (3, 23). In fact, Melk et al. (24) have recently reported that the *T869C* polymorphism in *TGF-β1* was significantly associated with TGF-β1 mRNA expression in normal kidney but not in kidneys from chronic allograft nephropathy. Although it is important to prove the direct association between TGF-β1 gene variation and its expression in local renal tissue, in this study, we could not measure the TGF-β1 mRNA expression in the renal tissue, because this study was retrospective. In addition, it is assumed that immunohistological study is not sufficient for providing convincing and quantitative data, partly because of the large interglomerular variation.

The *T869C* and *C-509T* polymorphisms in *TGF-β1* were specifically associated with marked proteinuria in terms of clinical manifestations of IgAN. The incidence of proteinuria of 1.0 g/day or more was significantly higher in patients with *CC* genotype than in those with other genotypes of both *C-509T* and *T869C* polymorphisms. Moreover, *C-C* haplotype of these polymorphisms was significantly more frequent in patients with urinary protein excretion of 1.0 g/day or more than in those with less proteinuria. We have no data available indicating that the increased amount of urinary protein in *–509CC* and *869CC* genotype was due to increased activity of TGF-β1 in local tissue. However, there is evidence that, in glomerular podocytes, TGF-β1 increases the production of vascular endothelial growth factor (VEGF) (25), which has a role in enhancing vascular permeability via nitric oxide and prostacyclin (26), as well as in proteinuria (27, 28). In addition to the effect on VEGF expression, TGF-β1 has a substantial effect on collagen synthesis in glomerular podocytes. A relative increase in the α3-chain of collagen IV, which may alter the structure of the GBM and affect its function as a filtration barrier, has been reported in podocytes in response to TGF-β1 (25).

In the histopathological assessment, the associations of the *TGF-β1* genotypes were detected only with mesangial cell proliferation, whereas no significant association was observed with respect to any other histopathological features including mesangial matrix increase and interstitial fibrosis. We could not clarify the underling mechanism in which the association was restricted to mesangial cell proliferation. We hypothesized that the polymorphisms in *TGF-β1* would predominantly associate with mesangial matrix increase and interstitial fibrosis, because the expression of TGF-β1 has mainly been reported to be associated with extracellular matrix expansion and interstitial fibrosis (29). However, in addition, TGF-β1 is known to have dual effects on the proliferation of a variety of cells. Indeed, TGF-β1 at a low concentration promotes mesangial cell proliferation and at a high concentration suppresses it *in vitro* (30). Therefore, our findings suggest that both the *CC* genotype of *C-509T* and *T869C* polymorphisms of *TGF-β1* are associated with higher activity of TGF-β1 in glomeruli with inflammatory injury than in other genotypes, but the increased activity is the extent to which mesangial cell proliferation is stimulated. Alternatively, TGF-β1 also up-regulates production of platelet-derived growth factor (PDGF) (31), and in the kidney of TGF-β1 gene-transfected rats, mesangial cell proliferation increased with extracellular matrix expansion (32). Because PDGF

stimulates the proliferation of mesangial cells (33, 34), the present result may reflect the effect of TGF-β1 on PDGF expression in glomeruli. There is also a possibility that the influences of TGF-β1 on mesangial matrix increase and interstitial fibrosis were less detectable than mesangial cell proliferation in the method, which we employed for the assessment of histopathological changes. In fact, both scores for mesangial matrix increase and interstitial fibrosis were consistently, but not significantly, higher in the *CC* genotype of *C-509T* and *T869C* polymorphisms of *TGF-β1*. Although this study could not reveal the functional significance of the gene polymorphisms, the findings of this study suggest that both the *C-509T* and *T869C* polymorphisms have a direct or indirect influence on the transcriptional activity of the *TGF-β1* gene. There is also a possibility that these gene polymorphisms may be in linkage disequilibrium with an undefined gene variation, which affects the TGF-β1 activity in glomerular inflammatory injury.

In conclusion, the present results indicate that *CC-509* and *CC869* genotypes, as well as *C-C* haplotype, of TGF-β1 gene polymorphisms are specifically associated with marked proteinuria and increased mesangial cell proliferation, both of which are risk factors of progression to ESRD in Japanese patients with IgAN. Although a prospective randomized controlled study with a long-term observation is needed to establish the prognostic significance of these gene polymorphisms, this translational approach supports a concept that components in the secretion/activation of TGF-β1 may be a target of therapeutic strategies in the future.

References

1. Okuda S, Languino LR, Ruoslahti E, Border WA. Elevated expression of transforming growth factor-beta and proteoglycan production in experimental glomerulonephritis. Possible role in expansion of the mesangial extracellular matrix. *J Clin Invest* 1990: **86**: 453–62.
2. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med* 1994: **331**: 1286–92.
3. Border WA, Ruoslahti E. Transforming growth factor-beta in disease: the dark side of tissue repair. *J Clin Invest* 1992: **90**: 1–7.
4. Bottinger EP, Bitzer M. TGF-beta signaling in renal disease. *J Am Soc Nephrol* 2002: **13**: 2600–10.
5. van Vlasselaer P, Punnonen J, de Vries JE. Transforming growth factor-beta directs IgA switching in human B cells. *J Immunol* 1992: **148**: 2062–7.
6. Awad MR, El-Gamel A, Hasleton P, Turner DM, Sinnott PJ, Hutchinson IV. Genotypic variation in the transforming growth factor-beta1 gene: association with transforming growth factor-beta1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. *Transplantation* 1998: **66**: 1014–20.
7. Grainger DJ, Heathcote K, Chiano M et al. Genetic control of the circulating concentration of transforming growth factor type beta1. *Hum Mol Genet* 1999: **8**: 93–7.
8. Levy M, Berger J. Worldwide perspective of IgA nephropathy. *Am J Kidney Dis* 1988: **12**: 340–7.
9. Radford Jr MG, Donadio Jr JV, Bergstralh EJ, Grande JP. Predicting renal outcome in IgA nephropathy. *J Am Soc Nephrol* 1997: **8**: 199–207.
10. Maisonneuve P, Agodoa L, Gellert R et al. Distribution of primary renal diseases leading to end-stage renal failure in the United States, Europe, and Australia/New Zealand: results from an international comparative study. *Am J Kidney Dis* 2000: **35**: 157–65.
11. Hsu SI, Ramirez SB, Winn MP, Bonventre JV, Owen WF. Evidence for genetic factors in the development and progression of IgA nephropathy. *Kidney Int* 2000: **57**: 1818–35.
12. Galla JH. Molecular genetics in IgA nephropathy. *Nephron* 2001: **88**: 107–12.
13. Ohtsuka T, Yamakage A, Yamazaki S. The polymorphism of transforming growth factor-beta1 gene in Japanese patients with systemic sclerosis. *Br J Dermatol* 2002: **147**: 458–63.
14. Watanabe Y, Kinoshita A, Yamada T et al. A catalog of 106 single-nucleotide polymorphisms (SNPs) and 11 other types of variations in genes for transforming growth factor-beta1 (TGF-beta1) and its signaling pathway. *J Hum Genet* 2002: **47**: 478–83.
15. Baan CC, Balk AH, Holweg CT et al. Renal failure after clinical heart transplantation is associated with the TGF-beta 1 codon 10 gene polymorphism. *J Heart Lung Transplant* 2000: **19**: 866–72.
16. Lacha J, Hubacek JA, Potmesil P, Viklicky O, Malek I, Vitko S. TGF-beta I gene polymorphism in heart transplant recipients-effect on renal function. *Ann Transplant* 2001: **6**: 39–43.
17. Yamada Y, Miyauchi A, Takagi Y, Tanaka M, Mizuno M, Harada A. Association of the C-509→T polymorphism, alone of in combination with the T869→C polymorphism, of the transforming growth factor-beta1 gene with bone mineral density and genetic susceptibility to osteoporosis in Japanese women. *J Mol Med* 2001: **79**: 149–56.
18. Yamada Y, Miyauchi A, Goto J et al. Association of a polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to osteoporosis in postmenopausal Japanese women. *J Bone Miner Res* 1998: **13**: 1569–76.
19. Yokota M, Ichihara S, Lin TL, Nakashima N, Yamada Y. Association of a T29→C polymorphism of the transforming growth factor-beta1 gene with genetic susceptibility to myocardial infarction in Japanese. *Circulation* 2000: **101**: 2783–7.
20. Suzuki S, Sato H, Kobayashi H et al. Comparative study of IgA nephropathy with acute and insidious onset. Clinical, laboratory and pathological findings. *Am J Nephrol* 1992: **12**: 22–8.
21. Wood NA, Thomson SC, Smith RM, Bidwell JL. Identification of human TGF-beta1 signal (leader) sequence polymorphisms by PCR-RFLP. *J Immunol Methods* 2000: **234**: 117–22.
22. Slatkin M. Linkage disequilibrium in growing and stable populations. *Genetics* 1994: **137**: 331–6.
23. Taniguchi Y, Yorioka N, Masaki T, Asakimori Y, Yamashita K, Yamakido M. Localization of transforming growth factors beta1 and beta2 and epidermal growth factor in IgA nephropathy. *Scand J Urol Nephrol* 1999: **33**: 243–7.

24. Melk A, Henne T, Kollmar T et al. Cytokine single nucleotide polymorphisms and intrarenal gene expression in chronic allograft nephropathy in children. *Kidney Int* 2003: **64**: 314–20.
25. Iglesias-de la Cruz MC, Ziyadeh FN, Isono M et al. Effects of high glucose and TGF-beta1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes. *Kidney Int* 2002: **62**: 901–13.
26. Murohara T, Horowitz JR, Silver M et al. Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin. *Circulation* 1998: **97**: 99–107.
27. Cha DR, Kim NH, Yoon JW et al. Role of vascular endothelial growth factor in diabetic nephropathy. *Kidney Int Suppl* 2000: **77**: S104–12.
28. Hovind P, Tarnow L, Oestergaard PB, Parving HH. Elevated vascular endothelial growth factor in type 1 diabetic patients with diabetic nephropathy. *Kidney Int Suppl* 2000: **75**: S56–61.
29. Yoshioka K, Takemura T, Murakami K et al. Transforming growth factor-beta protein and mRNA in glomeruli in normal and diseased human kidneys. *Laboratory Invest* 1993: **68**: 154–63.
30. Mene P, Simonson MS, Dunn MJ. Physiology of the mesangial cell. *Physiol Rev* 1989: **69**: 1347–424.
31. Roberts AB, Sporn MB. Regulation of endothelial cell growth, architecture, and matrix synthesis by TGF-beta. *Am Rev Respir Dis* 1989: **140**: 1126–8.
32. Isaka Y, Fujiwara Y, Ueda N, Kaneda Y, Kamada T, Imai E. Glomerulosclerosis induced by in vivo transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 1993: **92**: 2597–601.
33. Betsholtz C, Raines EW. Platelet-derived growth factor: a key regulator of connective tissue cells in embryogenesis and pathogenesis. *Kidney Int* 1997: **51**: 1361–9.
34. Floege J, Eng E, Young BA et al. Infusion of platelet-derived growth factor or basic fibroblast growth factor induces selective glomerular mesangial cell proliferation and matrix accumulation in rats. *J Clin Invest* 1993: **92**: 2952–62.

Kidney International, Vol. 65 (2004), pp. 15–24

Platelet-derived growth factor plays a critical role to convert bone marrow cells into glomerular mesangial-like cells

AKIRA SUZUKI,[1] HIROTSUGU IWATANI,[1] TAKAHITO ITO, ENYU IMAI, MASARU OKABE, HIROYUKI NAKAMURA, YOSHITAKA ISAKA, MASAYA YAMATO, and MASATSUGU HORI

Department of Internal Medicine and Therapeutics, Osaka University School of Medicine, Suita, Osaka, Japan; Osaka University Genome Information Research Center, Suita, Osaka, Japan

Platelet-derived growth factor plays a critical role to convert bone marrow cells into glomerular mesangial-like cells.

Background. Despite increasing interest in bone marrow–derived stem cells, little is known about critical factors that determine their fates both in vitro and in vivo. Recently, we have reported that bone marrow is a reservoir for glomerular mesangial cells in rats. To find a key factor responsible for the differentiation of bone marrow–derived cells into mesangial cells, we established a new culture system of rat bone marrow, which is based on serial replating and differential attachment to collagen types I and IV.

Methods. Bone marrow cells that did not adhere to collagen type I within 24 hours were transferred to collagen type IV–coated dishes. Then, the cells attached to collagen type IV in the following 24 hours were maintained in the presence of 2% horse serum, 200 ng/mL of platelet-derived growth factor (PDGF)-BB, and 1 μmol/L of all-*trans* retinoic acid. In vivo effect of PDGF-B was also examined by introducing human PDGF-B gene into glomeruli.

Results. After cultivation under the above condition for 7 days, approximately 14% of cells expressed Thy-1 and desmin, both of which are markers for rat mesangial cells. Thy-1^{++}/desmin^{+} cells were stellate-shaped, and contracted in response to angiotensin II. When human PDGF-B gene was overexpressed in the glomeruli of chimeric rats whose bone marrow was transplanted from enhanced green florescent protein (EGFP) transgenic rats, the number of EGFP^{+} mesangial cells increased. This effect was canceled by prior introduction of a neutralizing molecule that is composed of PDGF receptor-β ligand binding site and IgG-Fc.

Conclusion. These results indicate that PDGF-B plays a critical role to direct bone marrow–derived cells toward mesangial-like cells both in vitro and in vivo.

Mesangial cells, often considered to be equivalent to pericytes, provide physical support for the glomerular capillary lumen of the kidney. These cells are located in the intraglomerular structure, extend out into the juxtaglomerular zone, and show a low rate of cell division in normal mature glomeruli [1]. Since the normal glomerular capillary is essential to keep efficient ultrafiltration of the plasma, loss of mesangial cells due to pathologic conditions such as glomerulonephritis and diabetic nephropathy leads to impaired renal function. The exact developmental origin of mesangial cells is unknown, but it is generally accepted that mesangial cells come from the mesenchymal blastema or metanephric mesenchyme containing immature mesenchymal cells. Alternatively, mesangial cells may be derived from vascular pericytes, vascular smooth muscle cells, or myofibroblasts of invading vasculatures during the nephrogenesis [2, 3]. It is noteworthy that mesangial cells are not found in avascular glomeruli of transfilter-induced mesenchymes, suggesting that vasculature or blood circulation communicating with other tissues such as bone marrow may be crucial for the establishment of the mesangial structure [4].

Bone marrow–derived cells, whose contribution to the healing process of glomerular diseases remains unknown, attract great interest because of their therapeutic potentials. It has been established that mesenchymal stem cells, which are derived from bone marrow, have a potential to differentiate into different lineages in response to different environments [5, 6]. By tagging bone marrow–derived cells with enhanced green fluorescent protein (EGFP), we have recently reported that the adult bone marrow carries stem cells for glomerular mesangial cells [7, 8]. It can be expected that the mesangial stem cells share several basic properties with stem cells differentiating into other types of cells [9]. All stem cells need to proliferate in response to tissue damage, which should be promoted by the release of mitogens from injured tissue components, or by relief from inhibitors normally produced by healthy tissues. Subsequently, stem cells are engaged in the differentiation process. There is considerable evidence that restricted microenvironments, including types of

[1] Akira Suzuki and Hirotsugu Iwatani contributed equally to this study.

Key words: stem cell, GFP rat, PDGF, retinoic acid, mesangial cell.

Received for publication March 14, 2003
and in revised form August 4, 2003
Accepted for publication August 20, 2003

growth factors and cell-cell interactions, influence fates of multipotent stem cells and give them the competence to contribute to more specific lineages. So far, however, little is known about the events regulating the acquisition of the competence that is required for bone marrow–derived stem cells to differentiate into nonhematopoietic cells. Since nonhematopoietic stem cells in the bone marrow are spatially segregated from target organs that the stem cells contribute to build up or to regenerate, it is likely that the target organs secrete factors acting on nonhematopoietic stem cells.

The close relationship between mesangial cells and platelet-derived growth factor (PDGF)-B has been described from many points of view. PDGF-B is produced physiologically or pathologically by several renal cell types in vivo, which include mesangial, glomerular, and tubular epithelial, and endothelial cells in addition to infiltrating macrophages and platelets. PDGF-B, which mediates mitogenic and chemotactic effects on many mesenchymal types of cells, including vascular smooth muscle cells, fibroblasts, and mesangial cells in vitro [10], plays a pivotal role in the establishment of mature glomeruli that are structurally supported by mesangial cells. Mice deficient in PDGF-B or PDGF receptor (PDGFR)-β have primitive glomerular capillaries with glomerular epithelial cells and endothelial cells, but do not form the normal mesangium that is composed of mesangial cells and matrix proteins [11, 12]. Stromal cells surrounding the vascular cleft of the S-shaped body are likely to be the developmental precursor of mesangial cells because they express PDGFR-β [13, 14]. In our previous experiment, mesangiolysis evoked by the administration of anti-Thy-1 antibody was the key event to integrate bone marrow–derived cells as mesangial cells [8]. Several lines of evidence show that PDGF-B is responsible for the mesangial proliferation in this experimental glomerulonephritis. First, the expression of PDGF-B is up-regulated in this disease model [15]. Second, both the administration of PDGF-B neutralizing antibody [16] or oligonucleotide aptamers [17] and the overexpression of a chimeric molecule that is composed of the ligand binding site of PDGFR-β and IgG-Fc portion [18] suppress the proliferation of mesangial cells and inhibit the overproduction of extracellular matrix. By contrast, PDGF, when exogenously administered or overexpressed in glomeruli, induces mesangial proliferation [19, 20]. Moreover, human glomerular diseases with mesangial proliferation are accompanied by the up-regulation of PDGFRs [21, 22].

Here, by using a newly established in vitro system, we confirmed our previous in vivo results that the adult bone marrow carries stem cells for mesangial cells. In addition, we have identified that PDGF-B plays a key role for the recruitment and/or differentiation of bone marrow–derived mesangial stem cells both in vitro and in vivo.

METHODS

Animals

Wild-type Sprague-Dawley rats and transgenic Sprague-Dawley rats that express EGFP throughout their bodies (EGFP rat) were used [7, 8]. All rats weighing 150 to 180 g, anesthetized by intraperitoneal administration of pentobarbital, were subjected to the experiments. Procedures for the present study were approved by the Animal Committee at Osaka University School of Medicine.

Preparation of bone marrow and bone marrow transplantation

For culture, bone marrow cells were prepared from wild type male Sprague-Dawley rats [7, 8]. To establish bone marrow chimeric rats, bone marrow cells were harvested from male EGFP rats and were transplanted into wild-type female Sprague-Dawley rats as described previously [7, 8].

Cell culture

Freshly isolated bone marrow cells were resuspended in the growth medium [Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA)] supplemented with 10% fetal calf serum (FCS) (Gibco), 10% horse serum (Gibco), 0.5% chick embryo extract (Gibco), and 4% penicillin/streptomycin), and were seeded on collagen type I–coated dishes (BD PharMingen, San Diego, CA, USA) at a density of 5×10^6 cells/mL. Then, the cells were maintained in the growth medium at 5% CO2 and 37°C. After 24 hours, the cells that did not adhere to collagen type I were transferred to fresh collagen type IV–coated dishes (BD PharMingen). The supernatant containing cells that did not adhere to collagen type IV within another 24 hours were discarded by changing the medium. The cells that attached to collagen type IV were maintained for further 7 days in the growth medium or the differentiation medium [DMEM supplemented with 2% horse serum, 200 ng/mL rat PDGF-BB (R&D Systems, Minneapolis, MN, USA), 1 μmol/L all-*trans* retinoic acid (Sigma, Deisenhofen, Germany), and 4% penicillin/streptomycin]. The medium was changed every 3 days. Rat mesangial cells were obtained from 6-week-old Sprague-Dawley rats by the sieving method and were cultured in RPMI1640 (Sigma) supplemented with 20% FCS as described previously [8].

Immunocytochemistry and flow cytometric analysis

For immunocytochemistry, the culture cells were replated on chamber slides (Nunc, Naperville, IL, USA) and were fixed with ice-cold methanol for 5 minutes. Signals were detected by using fluorescein isothiocyanate (FITC)- or Texas Red-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA).

Primary antibodies except for mouse antirat Thy-1 monoclonal antibody (ox-7) were obtained from commercial sources: antidesmin (BioScience Products, Emmenbruecke, Switzerland), antipancytokeratin (Sigma), anti-RECA1 (CosmoBio, Tokyo, Japan), anti-Factor VIII (Neomarkers, Fremont, CA, USA), anticellular fibronectin (Abcam, Cambridge, UK), anti-Cd11b (Oxford Biotechnology, Oxford, UK), and anti-α-smooth muscle actin (Sigma). Ox-7 was provided by Dr. Seiichi Matsuo (Nagoya University, Nagoya, Japan). For flow cytometric analysis, adherent bone marrow cells on collagen types I or IV were rinsed with phosphate-buffered saline (PBS) twice and treated with 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) at room temperature. After 1 minute, the cells were dislodged by vigorous tapping the culture dishes, and trypsin was immediately neutralized with DMEM supplemented with 10% FCS. The dislodged cells were dispersed as possible by pipetting up and down, and clumps were removed by passing through 50 μm nylon mesh. It took more than 5 minutes to dislodge and disperse mesangial cells by treatment of 0.05% trypsin/0.02% EDTA at 37°C. To stain Thy-1 antigen, cells resuspended in PBS containing 2% FCS and 2% normal rat serum were reacted with anti-Thy-1 monoclonal antibody on ice for 1 hour. After washed in PBS containing 2% FCS and 2% normal rat serum three times, the cells were incubated with FITC-conjugated secondary antibody for 30 minutes on ice in the dark. Next, after washed in PBS, the cells were fixed in 4% paraformaldehyde/PBS on ice for 20 minutes in the dark and were permeabilized with 0.1% NP-40/1% bovine serum albumin(BSA)/PBS. Then, the cells were reacted with antidesmin polyclonal antibody on ice for 1 hour in the dark, which was followed by the incubation with phycoerythrin-conjugated secondary antibody and subsequent washes. Flow cytometric analysis was performed by using a FACSVantage SE (BD PharMingen).

Angiotensin II treatment

The cells were trypsinized and were seeded at a density of 1 to 10 × 10^3 per well in 24-well poly-L-lysine-coated plates. After 24 hours, the medium was replaced by Hanks' balanced salt solution supplemented with 10 mmol/L Hepes/NaOH (pH 7.4). Under a VHS video recording system, the cells were stimulated with 10 nmol/L of angiotensin II (Sigma) in the presence or absence of 1 nmol/L CV-11974 [an angiotensin II type 1 (AT_1) receptor antagonist] (Takeda Chemical Industries, Osaka, Japan). Captured images were converted to digital files by using video editing software (Adobe Premiere, Adobe System, Inc., San Jose, CA, USA).

Overexpression of PDGF-B in the kidney

Three weeks after the bone marrow transplantation, 200 μg of the plasmid pcDNA3 harboring human PDGF-B gene or the plasmid vector alone was injected into the left kidney via the left renal artery of the bone marrow chimeric rats. Immediately, the gene was introduced into glomeruli by using an electroporation technique [23]. This technique introduces an exogenous gene mainly into glomerular mesangial cells [23]. Neutralization of the effect of PDGF-B in rat glomeruli was performed as described previously [18]. Briefly, 200 μg of pCAGGS-PDGFR/Fc coding a hybrid molecule composed of PDGFR-β ligand binding site and IgG-Fc was transfected into the skeletal muscle of the right hindlimb of PDGF-B-transfected rats 24 hours before the transfection of PDGF-B by an electroporation technique [18]. One-week after the introduction of human PDGF-B gene, rats were thoroughly perfused with PBS followed by 10% buffered formalin via the abdominal aorta, and the kidneys were fixed for 3 hours in 10% buffered formalin at 4°C. The tissues were processed by using antibodies as follows: rabbit antidesmin polyclonal antibody, mouse antirat monocyte/macrophage monoclonal antibody (ED1)(Serotec, Ltd., Oxford, England), rabbit anti-green fluorescent protein (GFP) polyclonal antibody (Molecular Probe, Inc., Eugene, OR, USA), and corresponding secondary antibodies (Vector Laboratories, Burlingame, CA). Under a fluorescent microscope with the filter for Texas Red, glomeruli could be identified by observing faint background fluorescence even in the absence of Thy-1 staining.

Immunoblot analysis

Lower poles of the kidneys were homogenized in 2 volume of homogenizing buffer [20 mmol/L Tris HCl (pH 8.0)/140 mmol/L NaCl/1 mmol/L EDTA/5 mmol/L dithiothreitol (DTT)/protease inhibitor cocktail (Complete Mini™) (Roche Diagnostics, Mannheim, Germany). One hundred micrograms of the protein extract that was prepared from PDGF-transfected kidneys or control kidneys was subjected to immunoblot analysis by using antihuman PDGF-B polyclonal antibody (Ab-1) (R&D Systems). Signals were detected by using horseradish peroxidase-conjugated antirabbit IgG antibody and Renaissance™ chemiluminescence system (NEN, Boston, MA, USA).

RESULTS

Morphologic change of bone marrow cells in vitro

Crude preparation of bone marrow cells contains various types of cells, including marrow stromal cells, mesenchymal stem cells, and blood cells as well as hematopoietic stem cells. In order to roughly enrich immature stem cells from other types of cells, we followed and modified a method that is useful to enrich immature mesenchymal cells that are derived from skeletal muscle [24]. The growth medium, originally defined in that

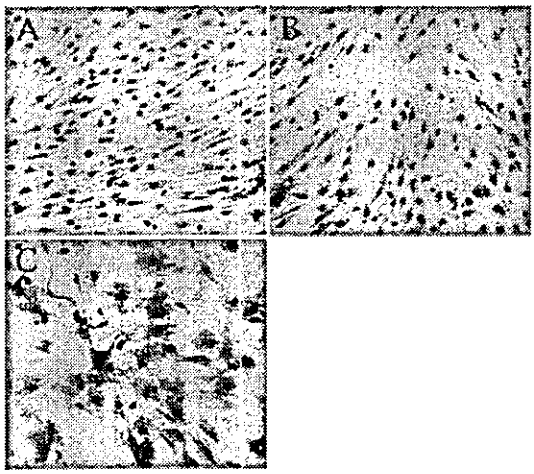

Fig. 1. Morphology of bone marrow cells in vitro. Rat bone marrow–derived cells were cultured as described in the **Methods** section. Then, the cells were fixed in ice-cold methanol and were stained with Giemsa. Light microscopic images are shown (×400). (*A*) Cells on collagen type I in the growth medium. (*B*) Cells on collagen type IV in the growth medium. (*C*) Cells on collagen type IV in the differentiation medium.

study, is capable of preserving the pluripotency of mouse skeletal muscle-derived mesenchymal stem cells for days [24].

In our experiments, 0.5% to 5% of bone marrow cells adhered to collagen type I, which depended on the preparation. The cells uniformly showed spindle-shape that was reminiscent of fibroblasts, proliferated fast in the growth medium, and created very dense culture (Fig. 1A). Next, the cells that did not adhere to collagen type I within the initial 24 hours were transferred to collagen type IV. Approximately, 0.2% to 3% of the cells adhered to collagen type IV in the following 24 hours, and were maintained in the growth or the differentiation medium. Although the cells on collagen type IV proliferated relatively slow in the growth medium when compared to the cells on collagen type I, both cells looked alike (Fig. 1A and B). We further focused our efforts on the cells that did not adhere to collagen type I but later adhered to collagen type IV, because stem cells for mesangial cells must be less adherent to reach the kidney via the circulation. When maintained in the differentiation medium, cells on collagen type IV ceased proliferation and subsequently changed into a wide range of shapes from spindle to stellate and from small to large (Fig. 1C). During the culture in the differentiation medium, a considerable number of cells fell into apoptosis and then detached. At the end of 7-day culture in the differentiation medium, simple calculation revealed that approximately 0.07% to 0.3% of the original bone marrow cells remained in the dishes. However, it was difficult to estimate how many numbers of or what types of cells contributed to the population shown in Figure 1C because the whole process is composed of the mixture of proliferation, apoptosis, and differentiation. All-*trans* retinoic acid induced extensive apoptosis of cells on collagen type IV when PDGF-BB was omitted (data not shown). By contrast, PDGF-BB alone was less effective to produce stellate cells, which suggests the supportive role of all-*trans* retinoic acid.

Expression of Thy-1 and desmin in cultured bone marrow cells

Since stellate cells in the differentiation medium were reminiscent of mesangial cells, the expression of Thy-1 and desmin was examined. Both molecules are well-established markers for rat mesangial cells in vitro [25, 26]. Flow cytometric analysis showed several distinct populations regarding the expression level of Thy-1 antigen (Fig. 2). Two of them, Thy-1–negative (Thy-1^-) and Thy-1–weakly positive (Thy-1^+) populations, were evident in the histogram of freshly isolated bone marrow cells and bone marrow cells that did not adhere to collagen type I (Fig. 2A and B). Another distinct group expressing Thy-1 antigen strongly (Thy-1^{++}) was seen in the cells that did not attach to collagen type I within 24 hours yet attached to collagen type IV in the following 24 hours (Fig. 2C and D). It is noteworthy that the culture in the differentiation medium produced more number of cells with stronger expression of Thy-1 than that in the growth medium. The increase of Thy-1^{++} cells was accompanied by the reciprocal decrease of Thy-1^- or Thy-1^+ cells, which suggested that Thy-1^{++} cells were converted from Thy-1^- and/or Thy-1^+ cells. Alternatively, the differentiation medium might support the preferential growth of Thy-1^{++} cells. Forward scatter analysis showed that Thy-$1^{+/++}$ cells were heterologous. Since a value of forward scatter roughly correlates with the size of a cell being analyzed, Thy-1^{++} cells seemed to contain cells larger than Thy-1^+ and Thy-1^- cells (Fig. 2F to I). Even cultured mesangial cells (passage 3) comprised heterologous types of cells in terms both of Thy-1 expression and of forward scatter (Fig. 2E and J). Because trypsinization was unavoidable to disperse cells before flow cytometric analysis and because cultured mesangial cells were more adhesive and cloggier than the cells on collagen type IV in the differentiation medium, we could not exclude a possibility that trypsinization differently affected the preservation of surface proteins such as Thy-1 and potentially intracellular proteins such as desmin. At least, however, digestion time for the cells from 2 minutes to 7 minutes gave essentially identical results (data not shown).

While 50 ng/mL of PDGF-BB was enough to protect cells from retinoic acid–induced apoptosis but not to produce Thy-1^{++} cells, the effect of 500 ng/mL of PDGF-BB was indistinguishable from that of 200 ng/mL of PDGF-BB as long as flow cytometric analysis was performed (data not shown). Therefore, there might be a critical concentration between 50 and 200 ng/mL.

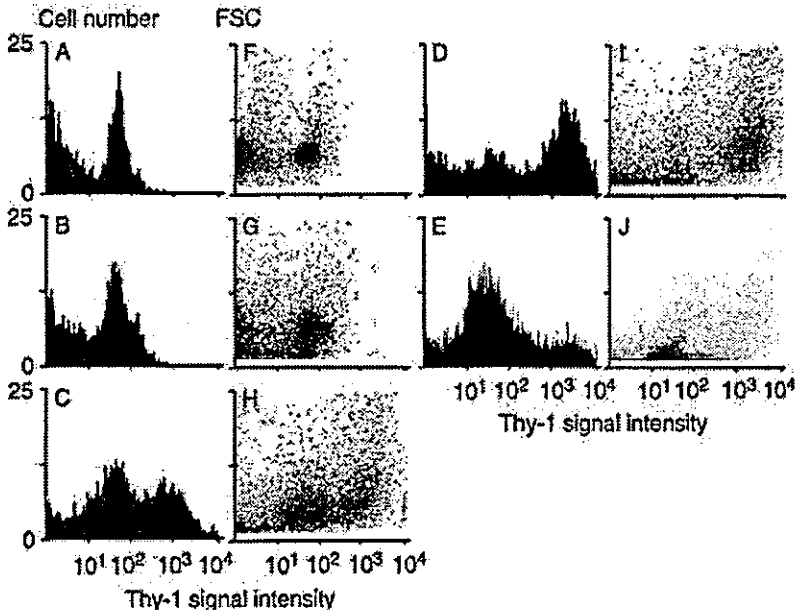

Fig. 2. Phenotypic changes of bone marrow culture. Rat bone marrow–derived cells and rat mesangial cells were stained with anti-Thy-1 antibody and were subjected to flow-cytometry. (*A* and *F*) Freshly isolated bone marrow cells. (*B* and *G*) Bone marrow cells that did not adhere to collagen type I within 24 hours (before replating to collagen type IV–coated dishes). (*C* and *H*) Bone marrow cells that adhered to collagen type IV (after 7-day culture in the growth medium). (*D* and *I*) Bone marrow cells that adhered to collagen type IV (after 7-day culture in the differentiation medium). (*E* and *J*) Rat mesangial cells [cultured in RPMI 1640/20% fetal calf serum (FCS), passage 3].

Fresh bone marrow preparation was completely desmin-negative, and only a small portion of cells that did not adhere to collagen type I within 24 hours expressed desmin weakly (Fig. 3A and B). In contrast, a significant number of the cells that attached to collagen type IV clearly expressed desmin (Fig. 3C and D). Among them, the expression level of desmin or Thy-1 was diverse, and most of Thy-1^+ or Thy-1^{++} cells did not express desmin (Fig. 3C and D). In the differentiation medium, however, approximately 14% of the cells on collagen type IV expressed Thy-1 and desmin strongly (Thy-$1^{+/++}$/desmin$^+$). This number was remarkably higher than the number of cells under the growth condition. In case of mesangial cells (passage 3), 45% of the cells expressed Thy-1 and desmin simultaneously (Fig. 3E). Again, these numbers might be underestimated because trypsinization would compromise the antigens.

Expression of Thy-1 and desmin was confirmed by immunocytochemistry (Fig. 4A). The expression level of Thy-1 was considerably diverse as shown in Figures 2 and 3. Here, we arbitrarily classified the cells into three classes again: Thy-1^-, Thy-1^+, and Thy-1^{++}. Thy-1^{++}desmin$^+$ cells were mostly flat and stellate. These cells were hardly found in the cells on collagen type I or in the cells on collagen type IV in the growth medium (data not shown). In consistence with flow cytometric analysis, Thy-$1^{+/++}$ cells were larger than Thy-1^- cells.

To further characterize the cells in the differentiation medium, expression of pancytokeratin, RECA1, factor VIII, cellular fibronectin, Cd11b, or α-smooth muscle actin was tested with mesangial cells as the control (Fig. 4B). Neither epithelial cells nor endothelial cells

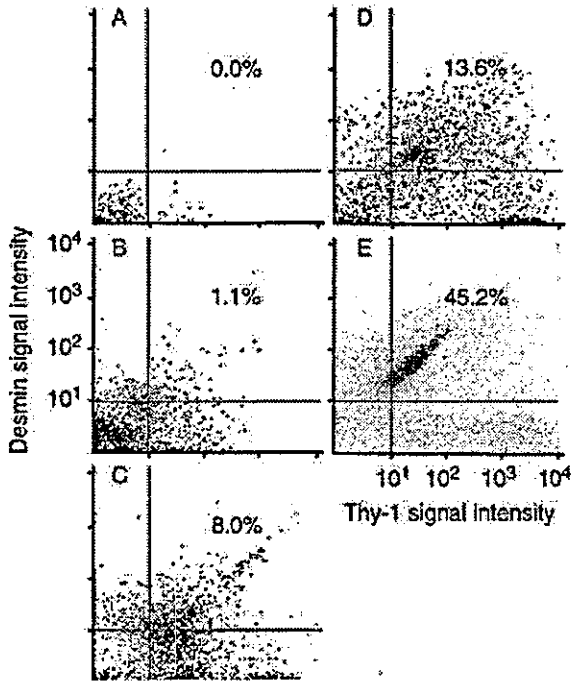

Fig. 3. Expression of Thy-1 and desmin. Cultured bone marrow–derived cells and rat mesangial cells were subjected to flow cytometric analysis after stained with anti-Thy-1 antibody and anti-desmin antibody. The percentage of cells expressing both Thy-1 and desmin is shown in each panel. (*A*) Freshly isolated bone marrow cells. (*B*) Bone marrow cells that did not adhere to collagen type I within 24 hours (before replating to collagen type IV–coated dishes). (*C*) Bone marrow cells that adhered to collagen type IV (after 7-day culture in the growth medium). (*D*) Bone marrow cells that adhered to collagen type IV (after 7-day culture in the differentiation medium). (*E*) Rat mesangial cells [cultured in RPMI 1640/20% fetal calf serum (FCS), passage 3].

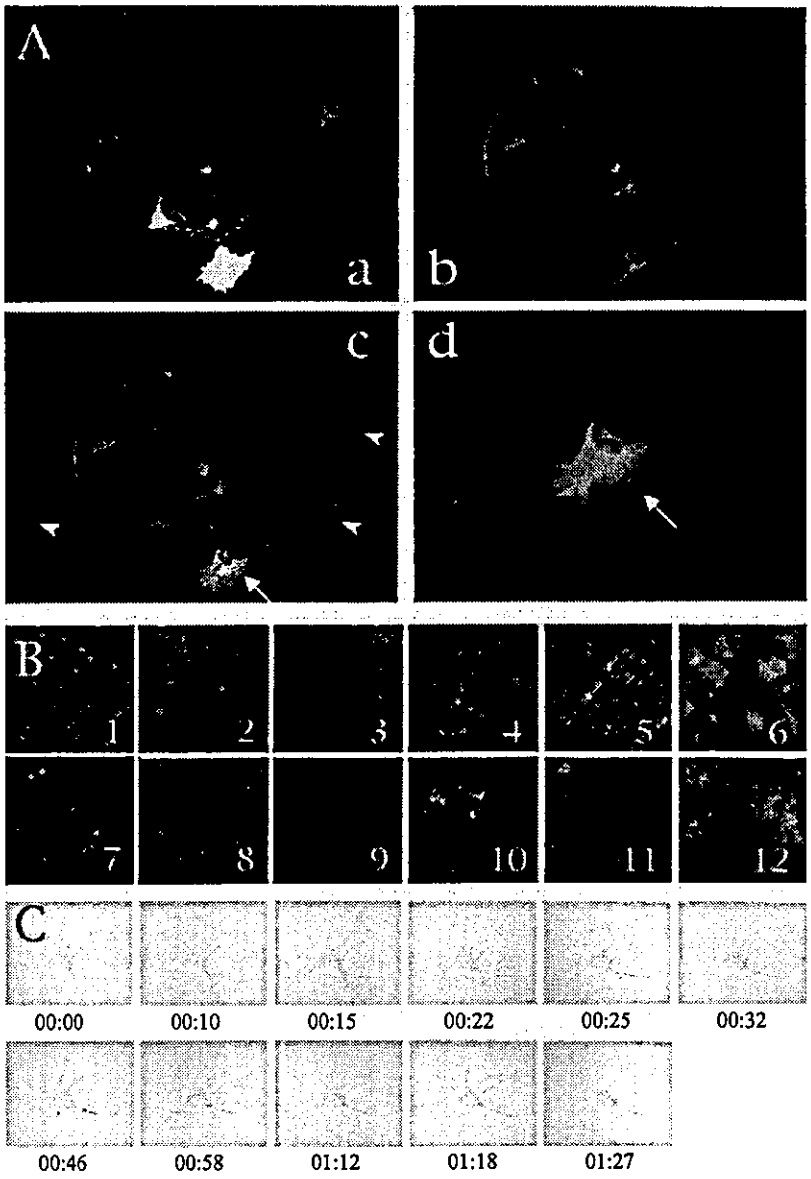

Fig. 4. Mesangial properties of differentiated cells. (*A*) Rat bone marrow–derived cells that were cultured on collagen type IV in the differentiation medium for 7 days were stained with anti-Thy-1 antibody and anti-desmin antibody. Immunocytochemical images are shown. (*A*) Panel a is Thy-1 (×200); panel b is desmin (×200); panel c is the merge (×200); and panel d is a larger image of the Thy-1^{++}desmin^{+} cell (×400). Various types of cells such as Thy-1^{+}desmin^{-} cell (yellow arrowhead), Thy-1^{-}desmin^{+} cell (white arrowhead), and Thy-1^{++}desmin^{+} cell (white arrow) are seen. (*B*) Rat bone marrow–derived cells and rat mesangial cells (passage 3) were stained with various antibodies (original magnification ×200). Panels 1 to 6 are cultured mesangial cells; panels 7 to 12 are bone marrow–derived cells on collagen type IV in the differentiation medium for 7 days. Panels 1 and 7 are pancytokeratin; panels 2 and 8 are RECA1; panels 3 and 9 are factor VIII; panels 4 and 10 are cellular fibronectin; panels 5 and 11 are Cd11b; and panels 6 and 12 are α-smooth muscle actin. (*C*) Time-lapse images of a cell that contracts in response to angiotensin II. Bone marrow–derived cells that adhered to collagen type IV were cultured in the differentiation medium for 7 days. Then, the cells were replated and then exposed to 10 nmol/L of angiotensin II. Representative sequential images are shown (total recording time was 1 minute and 27 seconds, QuickTime movie is available upon request). Time is indicated as min:second. Angiotensin II was added at 00:10.

were found in the cells as long as determined by the expression of pancytokeratin (Fig. 4B1 and B7), RECA1 (Fig. 4B2 and B8), and factor VIII (Fig. 4B3 and B9). Although a small number of round Cd11b-positive leukocytes existed (Fig. 4B5 and B11), they were rather remarkable in the mesangial culture (passage 3). The stellate cells in the differentiation medium clearly expressed cellular fibronectin (Fig. 4B4 and B10) and α-smooth muscle actin (Fig. 4B6 and B12), which highly resembled cultured mesangial cells.

Contraction of cultured bone marrow cells in response to angiotensin II

Mesangial cells in vitro have some characteristics that include stellate shape in culture and contraction in response to vasoactive peptides such as angiotensin II and vasopressin [27]. As shown in Figure 4C, angiotensin II induced dramatic contraction of the stellate cells. AT_1-receptor antagonist, CV-11974, completely abolished this effect of angiotensin II, and vehicle alone had no effect on the cells (data not shown). Following the video recording, the stellate cells that contracted in response to angiotensin II were fixed and stained. Finally, they were confirmed to express Thy-1^{++} or Thy-1^{+} and desmin (data not shown).

In vivo differentiation of bone marrow cells into mesangial cells

Our in vitro experiments revealed that PDGF-BB might play a role to convert bone marrow–derived cells

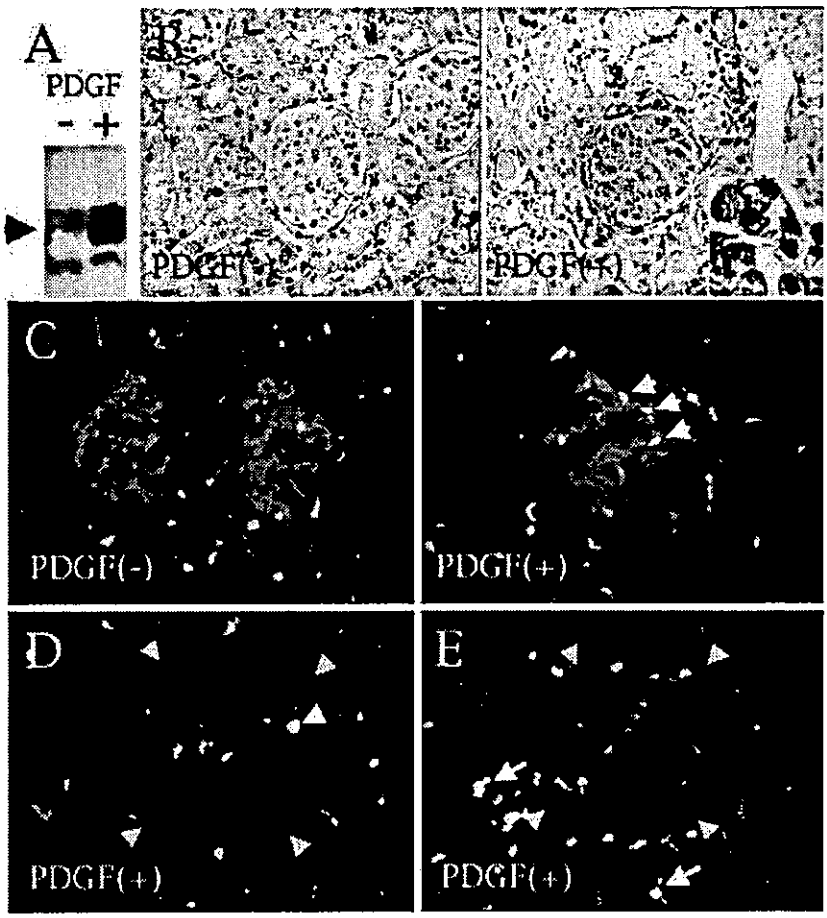

Fig. 5. Effects of the overexpression of human platelet-derived growth factor-B (PDGF-B) on glomeruli of chimeric rats. (*A*) Immunoblot analysis is shown. Protein extract obtained from mock-transfected kidneys or human PDGF-B–transfected kidneys was subjected to immunoblot analysis to see the expression of human PDGF-B. The arrow indicates PDGF-B. (*B*) Paraffin sections were stained with anti-GFP antibody (brown). Then, the sections were counterstained with hematoxylin-eosin (×400). PDGF(−) is mock-transfected kidneys (vector alone); PDGF(+) is PDGF-transfected kidneys. The inset shows representative brown cells with a higher magnification, which is indicated by yellow arrows. (*C*) Fixed frozen sections were stained with anti-Thy-1 antibody (red). Enhanced green florescent protein (EGFP) provides green (×400). Bone marrow–derived Thy-1–positive cells provide yellow color. Representative cells are indicated by yellow arrowheads. PDGF(−) is mock-transfected kidneys (vector alone); PDGF(+) is PDGF-transfected kidneys. (*D* and *E*) Fixed frozen sections prepared from PDGF-transfected kidneys were stained with anti-monocyte/macrophage (ED1) antibody or anti-CD45 antibody followed by Texas Red-conjugated secondary antibody (red). EGFP provides green. Glomeruli are surrounded by gray arrowheads for the reference. (D), ED1; (E), CD45. White arrowhead and arrow indicate $ED1^+EGFP^+$ cell and $CD45^+EGFP^+$ cell, respectively (×400).

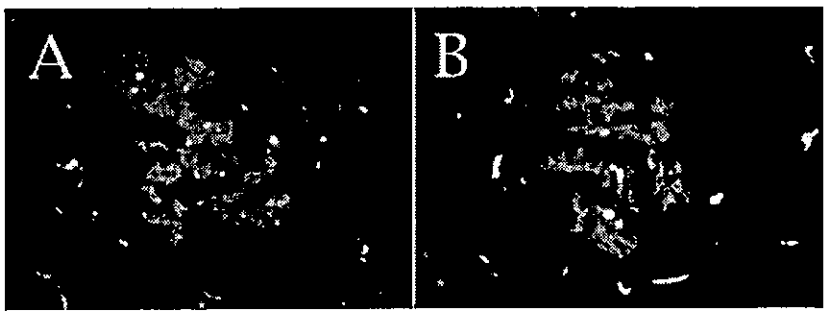

Fig. 7. Effect of the neutralization of human platelet-derived growth factor-B (PDGF-B) frozen sections were prepared from PDGF-transfected kidney with or without the neutralization by PDGFR/Fc and were stained with anti-Thy-1 antibody (red). Representative images are shown. (*A*) Human PDGF-B–transfected kidneys. (*B*) Human PDGF-B–transfected kidneys with the neutralization by PDGFR/Fc (×400).

into mesangial cells or at least into cells with mesangial properties. In order to examine the relevance of PDGF-B in vivo, human PDGF-B gene was transiently overexpressed in the left kidney of bone marrow chimeric rats by using the electroporation technique. This technique allows us to introduce a gene of interest mainly in glomeruli when the gene is administered via the renal artery [23]. Since normal glomeruli express collagen type IV, PDGF-transfected glomeruli would produce a condition at least partly mimicking the in vitro culture condition in the differentiation medium. One week after the electroporation, proliferation of mesangial cells in response to overexpressed PDGF-B was compatible with our previous report that exogenous PDGF-B gene induced mesangial proliferation when transfected to glomeruli by hemagglutinating virus of Japan (HVJ) liposome (Fig. 5B)[20]. The increase of bone marrow–derived $Thy\text{-}1^+$ cells (Fig. 5C, yellow cells) was observed in the PDGF-transfected glomeruli without significant infiltration of macrophages or $CD45^+$ blood cells (Fig. 5D and E). These cells were identified as bone marrow–derived mesangial cells according to our previous methods [8]. Each glomerulus had a quite different yet significant number of bone marrow–derived mesangial cells, which

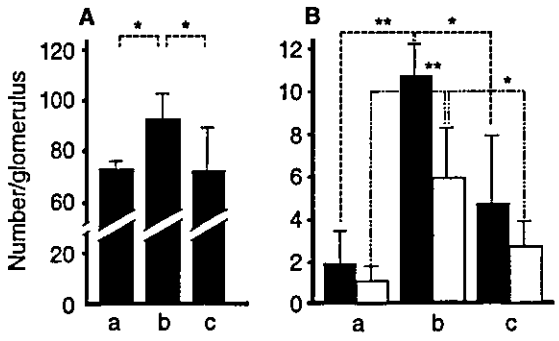

Fig. 6. Number of cells in platelet-derived growth factor (PDGF)-transfected glomeruli of chimeric rats. (*A*) The number of glomerular cells was counted by staining nuclei of frozen sections with 4′, 6 diamidino-2-phenylindole (DAPI). The results are shown as mean ± SD after 50 glomerular sections were counted. Lane a is mock-transfected kidneys; lane b is human PDGF-B–transfected kidneys; and lane c is human PDGF-B–transfected kidneys with the neutralization by PDGFR/Fc. (*B*) The numbers of enhanced green florescent protein-positive ($EGFP^+$)-glomerular cells (■) and $EGFP^+Thy\text{-}1^+$-glomerular cells (□) were counted. The results are shown as mean ± SD after 50 glomerular sections were counted. Lane a is mock-transfected kidneys; lane b is human PDGF-B-transfected kidneys; and lane c is human PDGF-B-transfected kidneys with the neutralization by PDGFR/Fc. Statistical significance was analyzed by *t* test, and is designated by asterisks ($^*P < 0.05$, $^{**}P < 0.01$).

presumably reflected the different transfection/expression level of human PDGF-B among the glomeruli (Fig. 6).

When a PDGF-neutralizing molecule, PDGFR-β ligand-binding domain fused with IgG-Fc was overexpressed in the skeletal muscle of the hindlimb of the chimeric rats 24 hours prior to the introduction of human PDGF-B, the increase of bone marrow–derived $Thy\text{-}1^+$ cells were prevented (Figs. 6 and 7).

DISCUSSION

We demonstrated here that bone marrow–derived cells can differentiate into mesangial-like cells in response to PDGF-BB, retinoic acid, and collagen type IV in vitro. These cells were identified mainly by the expression of Thy-1 and desmin, and by the appearance. Bone marrow–derived $Thy\text{-}1^{++}/desmin^+$ cells seemed more mesangial-like because they were stellate and contracted in response to angiotensin II. We, however, could not conclude that bone marrow–derived Thy-1/desmin-double positive cells were equal to cultured mesangial cells at least in terms of the expression level of Thy-1 (Fig. 2). Although about half of the cultured mesangial cells express both Thy-1 and desmin (Fig. 3), most of them seemed to be $Thy\text{-}1^+$ but not $Thy\text{-}1^{++}$ (Fig. 2). At the present moment, we do not know the detail of what makes the difference between $Thy\text{-}1^+/desmin^+$ cells and $Thy\text{-}1^{++}/desmin^+$ cells, or how different their properties are. Because both types of cells were observed even in cultured mesangial cells, the expression level of Thy-1 in culture might not matter as a property of mesangial cells. Uncharacterized cells such as fibroblasts also existed in the bone marrow culture. Probably, the expected percentage of mesangial stem/precursor cells in the bone marrow culture must be lower than the percentage of $Thy\text{-}1^{++}/desmin^+$ cells.

We also found that PDGF-B whose local concentration was increased by an in vivo gene transfer technique promoted the integration of bone marrow–derived mesangial cells in the glomeruli. These in vitro and in vivo data strongly support our previous result that bone marrow is a reservoir for mesangial cells [8], and highly suggest that PDGF-BB is an important factor for the differentiation. Various types of cells were produced in the differentiation medium, which is reminiscent of the fact that a single factor can induce differentiation of embryonic stem cells in vitro but produce a heterogeneous population [28]. Reportedly, effects of PDGF on cells seem to be modified by environmental factors. Functions of the PDGF-B/PDGFR-β system depend on microenvironments. The function served by the PDGF-B/PDGFR-β system in the reactive connective tissue formation is dissociated from that served during development of mesodermal lineages [29, 30]. PDGF (1 to 25 ng/mL) is shown to facilitate proliferation of bone marrow stromal cells, which are expected to comprise mesenchymal stem cells [31, 32]. However, although small yet significant amount of PDGF must be provided by FCS, horse serum and/or chicken embryo extract in the growth medium of our experiments, the medium did hardly convert bone marrow cells into mesangial-like cells. We used 200 ng/mL of PDGF-BB in the differentiation medium. Taking the effect of in vivo PDGF-B overexpression into consideration, we may assume that the conversion of bone marrow–derived cells into mesangial-like cells required higher concentration of PDGF. It is also possible that unknown factors that are included in FCS or chicken embryo extract in the growth medium interfere with the action of PDGF-BB.

It is not known yet whether human PDGF-B that was overexpressed in the rat glomeruli reached the bone marrow and induced the recruitment of stem cells. It is rather unlikely. Currently, we think that the following hypothesis is more plausible. Bone marrow–derived stem cells in circulation lodge in the interstitial area of the kidney. Then, in response to PDGF-BB that is locally up-regulated, the cells are recruited to glomeruli and are promoted to become mesangial cells. PDGF might function both as a chemoattractant within a short distance and as a differentiation factor, which remains to be clarified. We identified bone marrow–derived mesenchymal cells in the renal interstitium of bone marrow chimeric rats [7, 8]. Stem cells with mesenchymal multipotency are shown to enter the circulation [33]. Experiments to prove whether functional bone marrow–derived stem cells reside in the kidney are currently under way.

Microenvironment mentioned above includes matrix proteins. In this study, we fractionized bone marrow cells by serial replating in combination with collagen types I and IV. Collagen type IV was chosen because it is the major matrix protein of the normal mesangium and protects cultured mesangial cells from apoptosis while collagen type I that is overexpressed in diseased glomeruli does not promote the survival of mesangial cells [34]. Differential adhesion to substratum or matrix seems to be a useful approach to separate mesenchymal stem cells from differentiated cells. Lee et al [24] purified cells with mesenchymal multipotency from skeletal muscle by serial replating onto collagen type I–coated dishes. Cells with similar characteristics are also obtained from the bone marrow [35]. It is premature to say that the lower affinity to specific matrix proteins is a definitive trait of tissue stem cells in vitro. Nevertheless, the trait seems to be favorable if bone marrow–derived stem cells are recruited to the glomeruli in vivo.

The most primitive hematopoietic stem cells are $CD34^-$. Recently, murine hematopoietic stem cells that do not express CD34 differentiate into mesangial cells in vivo [36]. However, it is reported that both $CD34^+$ and $CD34^-$ stem cells can convert to $CD34^-$ and $CD34^+$ cells, respectively [37, 38]. Another type of stem cells in the bone marrow, mesenchymal stem cells, can give rise to multiple mesenchymal components such as chondrocytes, osteoblasts, and myoblasts [6]. This type of stem cells belongs to bone marrow stromal cells that produce various growth factors and also interact with hematopoietic stem cells at intercellular level [39]. Mesenchymal stem cells make an adherent monolayer of fibroblast-like cells on plastic dishes, and therefore look different from classical hematopoietic stem cells. Interestingly, plastic-adherent $CD34^{-/low}$ hematopoietic stem cells can produce both $CD34^+$ cells with hematopoietic activity and cells with mesenchymal plasticity [40, 41]. At the present moment, we have no data to tell what type of cells, $CD34^+$ or $CD34^-$, was converted into mesangial-like cells in vitro. Reyes and Verfaillie [42] and Jiang et al [43] have recently established multipotent adherent cells from bone marrow of human, mice, and rats. The cells, called multipotent adult progenitor cells, can proliferate without senescence in vitro and can contribute to all three germ layers in vivo, including hematopoietic lineage. After all, it seems to be difficult to distinguish stem cells with mesenchymal multipotency from the most primitive hematopoietic stem cells.

The significance of all-*trans* retinoic acid was uncertain. Basically, vitamin A plays indispensable roles for the nephrogenesis [44], and high concentration of all-*trans* retinoic acid induces the in vitro formation of pronephros from the animal cap of *Xenopus* eggs [45]. All-*trans* retinoic acid enhances the ex vivo maintenance of long-term repopulating stem cells [46], and stimulates transcription of PDGFR-α in a certain type of cells [47]. However, there was no significant change in the number of bone marrow–derived Thy-1^+ cells in the glomeruli even when all-*trans* retinoic acid (10 mg/kg) was subcutaneously administered for 7 days from the day of the electroporation (data not shown). Although endogenously produced all-*trans* retinoic acid might be sufficient, we have no answer yet.

Events of cell fusion should be considered especially when one performs fate mapping of given cells. Y chromosome fluorescent in situ hybridization (FISH) might be useful to detect the fusion when applied in combination with cells that are simultaneously labeled with other methods. Masuya et al [36] successfully performed Y chromosome FISH to exclude cell fusion in their male-to-male transplantation mice. They concluded that hematopoietic stem cell–derived mesangial cells in vivo are not due to fusion of two cells because only a single Y chromosome can be detected in $EGFP^+$ intraglomerular cells. However, at least in rats, we failed to obtain convincing signals by the combination of FISH and EGFP staining. Instead, our data that significant number of bone marrow–derived cells can be converted into mesangial-like cells in vitro under the specific condition strongly support that the generation of bone marrow–derived mesangial (-like) cells resulted from differentiation, not from the event of cell fusion.

CONCLUSION

We have successfully converted rat bone marrow–derived cells into mesangial-like cells, which share several properties with mesangial cells. Our in vitro and in vivo data indicate that PDGF-B plays a critical role to orchestrate the event.

ACKNOWLEDGMENTS

Human PDGF-B gene was obtained from American Type Culture Collection (originally deposited by Dr. Robert Gallo). We thank Dr. Seiichi Matsuo (Nagoya University, Nagoya, Japan) for providing human PDGF-B gene and anti-Thy-1 antibody. We also thank Naoko Horimoto for technical assistance. CV-11974 (2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid, active metabolite of Candesartan cilexetil) was kindly provided by Takeda Chemical Industries, Ltd., Osaka, Japan.

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, by a grant from Takeda Medical Research Foundation, and by a grant from the Osaka Kidney Foundation (OKF01-0009).

Reprint requests to Takahito Ito, M.D., Osaka University School of Medicine, Department of Internal Medicine and Therapeutics, Box A8, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.
E-mail: taka@medone.med.osaka-u.ac.jp

REFERENCES

1. PABST R, STERZEL RB: Cell renewal of glomerular cell types in normal rats. *Kidney Int* 24:626–631, 1983

2. Schlondorff D: The glomerular mesangial cells: An expanding role for a specialized pericytes. *FASEB J* 16:272–281, 1987
3. Latta H: An approach to the structure and function of the glomerular mesangium. *J Am Soc Nephrol* 2:S65–S73, 1992
4. Bernstein J, Cheng F, Roszka J: Glomerular differentiation in metanephric culture. *Lab Invest* 45:183–190, 1981
5. Caplan AI: Mesenchymal stem cells. *J Orthop Res* 9:641–650, 1991
6. Pittenger MF, MacKay AM, Beck SC, *et al*: Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147, 1999
7. Ito T, Suzuki A, Okabe M, *et al*: Application of bone marrow-derived stem cells in experimental nephrology. *Exp Nephrol* 9:444–450, 2001
8. Ito T, Suzuki A, Imai E, *et al*: Bone marrow is a reservoir of repopulating mesangial cells during glomerular remodeling. *J Am Soc Nephrol* 12:2625–2547, 2001
9. Morrison SJ, Shah NM, Anderson DJ: Regulatory mechanisms in stem cell biology. *Cell* 88:287–298, 1997
10. Heldin CH, Westermark B: Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* 79:1283–1316, 1999
11. Soriano P: Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev* 8:1888–1896, 1994
12. Leveen P, Pekny M, Gebre-Medhin S, *et al*: Mice deficient for PDGF-B show renal, cardiovascular, and hematological abnormalities. *Genes Dev* 8:1875–1887, 1994
13. Arar M, Xu YC, Elshihabi I, *et al*: Platelet-derived growth factor receptor beta regulates migration and DNA synthesis in metanephric mesenchymal cells. *J Biol Chem* 275:9527–9533, 2000
14. Alpers CE, Seifert RA, Hudkins KL, *et al*: Developmental patterns of PDGF B-chain, PDGF-receptor, and alpha-actin expression in human glomerulogenesis. *Kidney Int* 42:390–399, 1992
15. Iida H, Seifert R, Alpers CE, *et al*: Platelet-derived growth factor (PDGF) and PDGF receptor are induced in mesangial proliferative nephritis in the rat. *Proc Natl Acad Sci USA* 88:6560–6564, 1991
16. Johnson RJ, Raines E, Floege J, *et al*: Inhibition of mesangial cell proliferation and matrix expression in glomerulonephritis by antibody to platelet-derived growth factor. *J Exp Med* 175:1413–1416, 1992
17. Floege J, Ostendorf T, Janssen U, *et al*: Novel approach to specific growth factor inhibition in vivo: Antagonism of platelet-derived growth factor in glomerulonephritis by aptamers. *J Am Soc Nephrol* 10:169–179, 1999
18. Nakamura H, Isaka Y, Tsujie M, *et al*: Electroporation-mediated PDGF receptor-IgG chimera gene transfer ameliorates experimental glomerulonephritis. *Kidney Int* 59:2134–2145, 2001
19. Floege J, Eng E, Young BA, *et al*: Infusion of platelet-derived growth factor or basic fibroblast growth factor induces selective glomerular mesangial cell proliferation and matrix accumulation in rats. *J Clin Invest* 92:2952–2962, 1993
20. Isaka Y, Fujiwara Y, Ueda N, *et al*: Glomerulosclerosis induced by in vivo transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 92:2597–2601, 1993
21. Gesualdo L, Di Paolo S, Milani S, *et al*: Expression of platelet-derived growth factor receptors in normal and diseased human kidney. An immunohistochemistry and in situ hybridization study. *J Clin Invest* 94:50–58, 1994
22. Fellstrom B, Klareskog L, Heldin CH, *et al*: Platelet-derived growth factor receptors in the kidney—Upregulated expression in inflammation. *Kidney Int* 36:1099–1102, 1989
23. Tsujie M, Isaka Y, Nakamura H, *et al*: Electroporation-mediated gene transfer that targets glomeruli. *J Am Soc Nephrol* 12:949–954, 2001
24. Lee JY, Qu-Petersen Z, Cao B, *et al*: Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. *J Cell Biol* 150:1085–1099, 2000
25. Paul LC, Rennke HG, Milford EL, Carpenter CB: Thy1.1 in glomeruli of rat kidneys. *Kidney Int* 25:771–777, 1984
26. Yaoita E, Kazama T, Kawasaki K, *et al*: In vitro characteristics of rat mesangial cells in comparison with aortic muscle cells and dermal fibroblasts. *Virchows Arch* 49:285–294, 1985
27. Ausiello DA, Kreisberge JI, Roy C, Karnovsky MJ: Contraction of rat glomerular cells of apparent mesangial origin after stimulation with angiotensin II and arginine vasopressin. *J Clin Invest* 65:754–760, 1980
28. Schuldiner M, Yanuka O, Itskovitz-Eldor J, *et al*: Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 97:11307–11312, 2000
29. Crosby JR, Tappan KA, Seifert RA, Bowen-Pope DF: Chimera analysis reveals that fibroblasts and endothelial cells require platelet-derived growth factor receptor beta expression for participation in reactive connective tissue formation in adults but not during development. *Am J Pathol* 154:1315–1321, 1999
30. Crosby JR, Seifert RA, Soriano P, Bowen-Pope DF: Chimaeric analysis reveals role of Pdgf receptors in all muscle lineages. *Nat Genet* 18:385–388, 1998
31. Rosenfeld M, Keating A, Bowen-Pope F, *et al*: Responsiveness of the in vitro hematopoietic microenvironment to platelet-derived growth factor. *Leukemia Res* 9:427–434, 1985
32. Kuznetsov SA, Friedenstein AJ, Robey P: Factors required for bone marrow stromal fibroblast colony formation in vitro. *British J Hematol* 97:561–570, 1997
33. Orlic D, Kajstura J, Chimenti S, *et al*: Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci USA* 98:10344–10349, 2001
34. Mooney A, Jackson K, Bacon R, *et al*: Type IV collagen and laminin regulate glomerular mesangial cell susceptibility to apoptosis via beta(1) integrin-mediated survival signals. *Am J Pathol* 155:599–606, 1999
35. Hall FL, Han B, Kundu RK, *et al*: Phenotypic differentiation of TGF-beta1-responseive pluripotent premesenchymal prehematopoietic progenitor (P4 stem) cells from murine bone marrow. *J Hematol Stem Cell Res* 10:261–271, 2001
36. Masuya M, Drake CJ, Fleming PA, *et al*: Hematopoietic origin of glomerular mesangial cells. *Blood* 15: 2215–2218, 2003
37. Sato T, Laver JH, Ogawa M: Reversible expression of CD34 by murine hematopoietic stem cells. *Blood* 94:2548–2554, 1999
38. Zanjani ED, Almeida-Porada G, Livingston AG, *et al*: Human bone marrow $CD34^-$ cells engraft in vivo and undergo multilineage expression that includes giving rise to $CD34^+$ cells. *Exp Hematol* 26:353–360, 1998
39. Dorshkind K: Regulation of hematopoiesis by bone marrow stromal cells and their products. *Annu Rev Immunol* 8:111–137, 1990
40. Huss R, Hong DS, McSweeney PA: Differentiation of canine marrow cells with hematopoietic characteristics from an adherent stromal cell precursor. *Proc Natl Acad Sci USA* 92:748–752, 1995
41. Huss R, Lange C, Weissinger EM, *et al*: Evidence of peripheral blood-derived, plastic-adherent $CD34^{-/low}$ hematopoietic stem cell clones with mesenchymal stem cell. *Stem Cells* 18:252–260, 2000
42. Reyes M, Verfaillie CM: Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. *Ann N Y Acad Sci* 938:231–235, 2001
43. Jiang Y, Jahagirdar BN, Reinhardt RL, *et al*: Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418:41–49, 2002
44. Gilbert T, Merlet-Benichou C: Retinoids and nephron mass control. *Pediatr Nephrol* 14:1137–1144, 2001
45. Chan TC, Arizumi T, Asashima M: A model system for organ engineering: Transplantation of in vitro induced embryonic kidney. *Naturwissenschaften* 86:224–227, 1999
46. Purton LE, Bernstein ID, Collins SJ: All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. *Blood* 95:470–477, 2000
47. Wang C, Kelly J, Bowen-Pope DF, Stiles CD: Retinoic acid promotes transcription of the platelet-derived growth factor alpha-receptor gene. *Mol Cell Biol* 10:6781–6784, 1990

Kidney International, Vol. 65 (2004), pp. 1135–1144

PERSPECTIVES IN RENAL MEDICINE

Relationship between tonsils and IgA nephropathy as well as indications of tonsillectomy

YUANSHENG XIE, XIANGMEI CHEN, SHINICHI NISHI, ICHIEI NARITA, and FUMITAKE GEJYO

Kidney Center of PLA, Department of Nephrology, Chinese General Hospital of PLA, Beijing, China; and Division of Clinical Nephrology and Rheumatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Relationship between tonsils and IgA nephropathy as well as indications of tonsillectomy. Although there are many papers about IgA nephropathy (IgAN) and tonsils, respectively, reviews about the relationship between tonsils, tonsillitis, tonsillectomy, and IgAN are limited. In this review, we introduced the structure, development, and function of tonsils, difference of tonsils with and without IgAN, consistency of both tonsillar IgA and glomerular IgA, the effect of tonsil stimulation, tonsil infection, and tonsillectomy on IgAN showed some evidences in which tonsils were closely related to IgAN and polymeric IgA1 deposited in glomerular mesangium were at least in part of tonsillar origin. Tonsillectomy can improve the urinary findings, keep stable renal function, improve mesangial proliferation and IgA deposit, have a favorable effect on long-tern renal survival in some IgAN patients, and do not cause significant immune deficiency and do not increase incidence of the upper respiratory tract infections, and can be used as a potentially effective treatment. The indications of tonsillectomy in patients with IgAN include mainly the deterioration of urinary findings after tonsillar infection, mild or moderate renal damage. However, tonsillectomy may not be enough and may not change the prognosis in IgAN patients with marked renal damage.

Immunoglobulin A nephropathy (IgAN), that is, nephropathy with mesangial IgA-IgG deposits, was first reported by Berger and Hinglais in Frence in 1968 [1] and described by Berger in English in 1969 [2]. Studies for more than 30 years demonstrated that primary IgAN is an immune complex–mediated glomerulonephritis defined immunohistologically by the presence of glomerular IgA deposits [3]. It is now generally known to be the most common form of primary glomerulonephritis throughout the world [4–6]. Although primary IgAN was considered a benign condition for many years, it is now clear that a large number of cases eventually progress to renal failure [7–11]. Indeed, IgAN is the main cause of end-stage renal disease (ESRD) in patients with primary glomerular disease who require renal replacement therapy [12, 13]. However, the cause of primary IgAN, source of IgA deposited in glomeruli and the mechanism underlying mesangial IgA deposition in IgAN, is unclear and there is no effective treatment available for patients with IgAN [14].

The IgA deposited in glomerular mesangium in patients with IgAN appears to be exclusively of the IgA1 subclass [15] and IgA produced by tonsillar lymphcytes in patients with IgAN is mainly polymeric IgA1, about half of patients with IgAN their serum IgA levels increase [16] and tonsillectomy decreases the levels of serum IgA, suggesting there is any relationship between tonsils and IgAN. Recently, we demonstrated that the tonsillectomy has a favorable effect on long-term renal survival in patients with IgAN [17].

Although there are many papers about IgAN and tonsils, respectively, reviews about the relationship between tonsils, tonsillectomy, and IgAN are limited. In this review, we introduce the structure, development, and function of tonsils, difference of tonsils with and without IgAN, consistency of both tonsillar IgA and glomerular IgA, the effect of tonsil stimulation, tonsil infection, and tonsillectomy on IgAN, show some evidences in which tonsils were closely related to IgAN and polymeric IgA1 deposited in glomerular mesangium were at least in part of tonsillar origin, and present the indications of tonsillectomy in patients with IgAN.

STRUCTURE, DEVELOPMENT, AND FUNCTIONS OF TONSILS

Structure of tonsils

Human tonsils include the palatine tonsils, nasopharyngeal tonsil (adenoid), lingual tonsil and the tubal tonsils [18] (Fig. 1). The palatine tonsils are the largest ones in four types of tonsils in human beings. Histologically, tonsil tissues consist of four well-defined microcompartments,

Key words: tonsils, tonsillectomy, IgA nephropathy, treatment, indication.

Received for publication August 3, 2003
and in revised form September 28, 2003
Accepted for publication October 28, 2003

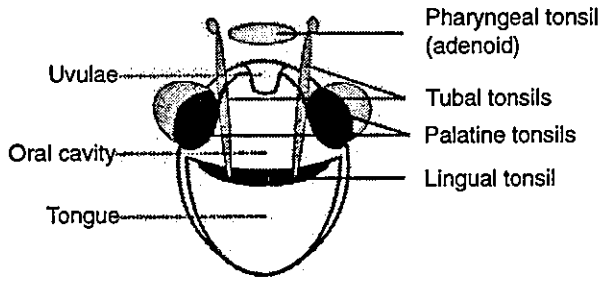

Fig. 1. Anatomy of the tonsils.

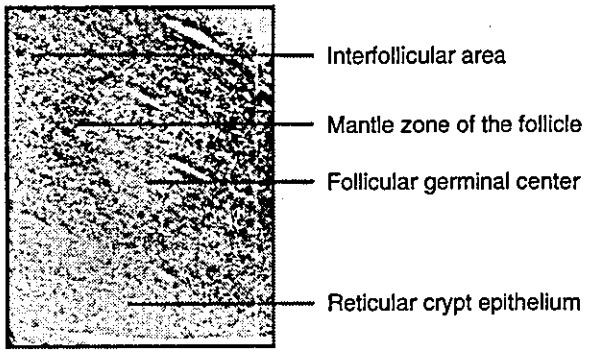

Fig. 2. Histologic structure of tonsils. The sample originated from tonsil tissues after tonsillectomy because of chronic tonsillitis in 8-year-old, male patient. The cellular nuclei of the section were stained with hematoxylin (original magnification ×50).

which all participate in the immune response: the reticular crypt epithelium, the interfollicular (extrafollicular) area, the mantle zone of lymphoid follicles, and the follicular germinal center [19] (Fig. 2). Cell biologically, immunocyts of tonsil tissues contain predominantly B cells (approximately 65%), approximately 30% $CD3^+$ T cells, and 5% macrophages. The T cells were primarily of the $CD4^+$ subset (approximately 80%) [20]. Quantitative immunohistochemistry reveals that IgG-containing B cells predominate in all lymphoid compartments, including follicles, extrafollicular areas, and reticular epithelium, whereas IgA cells are found predominantly in extrafollicular areas, especially subepithelial area, and IgM cells are in follicles. J chain is present within IgM and some IgA cells. The IgG:IgA:IgM class ratios of the overall tonsillar immunocyte population are 13:8:2. Cells containing IgD and IgE are rare [21]. In clinically normal tonsils, the overall percentage distribution of these cells is 65:30:3.5:1.2 for the IgG, IgA, IgM. and IgD classes, respectively. In recurrent tonsillitis, these figures are 53:39:4.7:4.4; in hyperplastic tonsillitis, 67:25:4.0:4.5; and in idiopathic tonsillar hyperplasia, 50:33:7.2:10, respectively [22]. In comparison with the clinically healthy tonsils, the number, the size of the germinal centers and the density of the immunocytes in tonsils are very large in the hyperplastic tonsils, large in chronic cryptic tonsillitis, but remarkably decreased in acute tonsillitis [23]. The study regarding the distribution and proportion of Ig subclasses producing cells in chronic tonsillitis show that the percentage ratios of IgG1:IgG2:IgG3:IgG4 were 53.1:35.9:4.7:6.3, respectively. Proportional ratios of IgA1:IgA2 are approximately 80:20 [24].

Development of tonsils

The development of the palatine tonsils starts during the 14th gestational week when the mesenchyme underlying the mucous membrane of the tonsillar cavity becomes invaded by mononuclear wandering cells. In fetuses of about the 16th gestational week epithelial crypts grow down into the connective tissue and are infiltrated by T lymphocytes. At the same time, precursors of interdigitating cells can be identified among the epithelial cells. Primary follicles develop in earlier fetal stages than in all other secondary lymphoid organs. They contain precursors of dendritic reticulum cells and lymphoid cells that belong to the B-cell line. These primary follicles may be considered as the first assemblage of B-cell regions in human fetal lymphoid tissue [25]. The formation of the follicular germinal centers reflecting B-cell activation by exogenous antigens takes place shortly after birth [26]. The immunohistochemical study show the morphometric features of tonsils below the age of 8 years are more active than those above the age of 8 years. Total number of IgA immunocytes is the highest at the age of 5 to 7 years with a decline by age. The serum IgA and salivary secretory IgA concentrations reach to adult's level at the age of 11 to 13 years. These results suggest that tonsils in preschool children are important as a local immunological defense mechanism [27].

Functions of tonsils

Tonsil tissues are located at the gateway of the respiratory and alimentary tract and belong to the mucosa-associated lymphoid tissue. The generation of B cells in the germinal centers of the tonsil is one of the most essential tonsillar functions. The major function of tonsils is as a first line of defense against viral, bacterial, and food antigens that enter the upper aerodigestive system. Secretory dimeric IgA produced by B cells has particular hydrophilic properties and is capable of preventing adsorption and penetration of bacteria and/or viruses into the upper respiratory tract mucosa [28]. With the uptake of antigen by microfold cells (membrane cell, M cells) present in the cryptepithelium a process is initiated, which ultimately results in the generation and dissemination of antigen-specific memory and mainly dimeric IgA-producing effector B lymphocytes. This process requires successful cognate interactions between antigen-presenting cells and lymphocytes and mutually between lymphocytes, which depend not only on antigen-specific

Table 1. Difference of tonsils with (+) and without (−) IgA nephropathy (IgAN)

Characteristic of tonsils	IgAN(+)	IgAN(−)	Reference number
T cell area (T nodules)	Expanded	Not expanded	29
Reticulization of crypt epithelium	Reduced	Not reduced	31
IgA cells:IgG cells	>1	<1	32–34
Polymeric IgA cells	Increased	Not changed	32–34
Polymeric IgA:IgA	Increased	Not changed	35
Follicular dendritic cells	IgA1+	IgA1−	36
J chain mRAN-positive cells	Increased	Not changed	37
Adhesion molecules CD31, CD54	Increased	Not changed	38
CD5+ B cells	Increased	Not changed	39

signals, but also on the expression of various complementary adhesion and costimulatory molecules [19]. In additional, Mitogen-triggered T cells from tonsils produced both of Th1- and Th2-type cytokines, clearly exhibiting their pluripotentiality for support of cell-mediated and antibody responses. The antigen-specific T cells produced interferon-gamma (INF-γ) and lower levels of interleukin-5 (IL-5). These results suggest that tonsils of the nasopharyngeal-associated lymphoreticular tissues represent a distinct component of the mucosal-associated lymphoreticular tissues with features of both systemic and mucosal compartments [20].

DIFFERENCE OF TONSILS WITH AND WITHOUT IgA NEPHROPATHY

There are significant differences in histologic structure, proportional ratios of cells, and cell adhesion molecules in tonsils with and without IgAN (Table 1).

Difference of tonsillar histologic structure

The enlarged primary T nodules in tonsils, which are defined as sum of the small areas of accumulating T lymphocytes and apparent nodule, composed predominantly of small T lymphocytes, were a characteristic feature of tonsils in patients with IgAN. Most T nodules in patients with IgAN were enlarged, especially in younger patients, and a few T nodules contain high endothelial venules and nonlymphoid cells. In contrast, T nodules in patients with habitual tonsillitis do not expand, and nonlymphoid cells and high endothelial venules are distributed peripherally around the nodules [29]. The basic structure and functional unit of reactive lymph nodes is composed of two separate T nodules and B-lymphoid follicles. These composite nodules play a major role in the triggering, helper T-cell–dependent stimulation and subsequent maturation of antigen-responsive B cells into antibody-secreting plasma cells [30]. With regard to his relationship between T-cell and B-cell domains, the enlarged primary T nodules reminds us that extrafollicular maturation of the stimulated B lymphocytes into plasma cells may occur more frequently in the tonsils of patients with IgAN than in patients with habitual tonsillitis. Another study demonstrated abnormal reticulization of tonsillar crypt epithelium in patients with IgAN. Tonsils of controls with recurrent tonsillitis or tonsillar hypertrophy showed well-developed reticular crypt epithelia with lymphoepithelial symbiosis, and the nonreticulated area was less than 7% of the total crypt epithelia per overall section. In IgAN tonsils, however, nonreticulated crypt epithelium was frequently observed and, in the advanced stage of IgAN, exceeded 50% of total crypt epithelia [31].

Difference of tonsillar cells

Primary IgAN is characterized by renal deposits of polymeric IgA (J chain-positive), the origin of which is not confirmed, yet. The study by Bene et al [32] showed that in controls with recurrent tonsillitis, IgG secreting cells were predominant (IgG secreting cells 65% and IgA plasma cells 29%), while in the IgAN patients, the plasma cells percentages was of an inversion (IgG 37% and IgA 56%). This increment in the IgA population was paralleled by an augmentation of the number of dimeric IgA secreting cells (75% of IgA plasma cells), stained both for cytoplasmic IgA and J chain [32]. The study by Nagy and Brandtzaeg [33] and a later multicenter study by Bene et al [34] also demonstrated a similar result. In additional, after 7 days of culture with pokeweed mitogen, the percentage of tonsillar cells producing polymeric IgA is significantly higher in the IgAN patients than in the controls suffering from chronic tonsillitis [35]. The IgA1 subclass was found in follicular dendritic cells (FDC) of the tonsil of IgAN patients, but not in FDC of non-IgAN controls. On the other hand, IgA2, IgG, IgM, and C3 did not show any differences in distribution between the two groups [36]. In situ hybridization (ISH) study for the detection of J chain mRNA within IgA plasma cells revealed J chain mRNA-positive cells were identified in germinal centres, and within the subepithelial and interfollicular zones of tonsils. Combined immunofluorescence and fluorescent ISH showed a greater proportion of J chain mRNA-positive interfollicular IgA cells in the patient tonsils compared with the controls. In additional, the finding of excess numbers of J chain-positive IgA-negative cells was found within germinal centers of tonsils in IgAN patients [37]. These results demonstrate immune abnormalities within the tonsil as a central feature of abnormal polymeric IgA biology in IgAN, which is in keeping with the hypothesis favoring a tonsil origin for the mesangial IgA present in their kidneys.

Abnormalities in the partition of IgA- and IgG-producing cells in the tonsils of patients with IgAN have been suggested to result from a dysregulation of cell

trafficking and homing through high endothelial venules in this lymphoid tissue. Study demonstrated a significant enhancement of cell adhesion molecules, CD31 and CD54, expression on high endothelial venules of tonsils from patients with IgAN compared with controls [38]. In additional, the number of $CD5^+$ B cells isolated from the tonsil germinal centers of IgAN patients is increased. These $CD5^+$ B cells are likely IgA1 antibody-producing cells. Moreover, these $CD5^+$ B cells show a reduced susceptibility to Fas-mediated apoptosis [39].

RELATIONSHIP BETWEEN TONSILLAR IgA AND GLOMERULAR IgA

Both IgA produced by tonsil cells and IgA deposited in glomerular mesangium with IgAN are mainly J chain-positive polymeric IgA [35, 37, 40, 41]. Studies demonstrated they were consistent in some cases. The antibodies eluted from renal tissues of patients with IgAN specifically bound with the nuclear regions of tonsillar cells. The binding of eluted antibodies and tonsillar cells was completely inhibited by the addition of antihuman IgA antisera, but not inhibited by human IgA myeloma proteins. The eluted antibodies bound with tonsillar cells from the same patients, but only 10% of them bound with the tonsillar cells obtained from other patients with IgAN. This result suggests that IgA antibodies deposited in glomeruli specifically bind with tonsillar cells obtained from patients with IgAN [42]. The study by Tokuda et al [43] offered another evidence of binding of IgA produced by tonsillar B lymphocytes to the glomerular mesangium of IgAN. They first made heterohybridoma cells of human tonsillar B lymphocytes from IgAN patient with mouse myeloma cells and cultured them. The culture medium was analyzed by Western blot analysis using antihuman IgA antibody, and both IgA1 and IgA2 were demonstrated to be produced. The specimens of the biopsied kidney tissue of IgAN were washed with 0.02 mol/L citrate buffer (pH 3.2) to remove deposited IgA from glomerulus. The specimens were then incubated with the culture media of hybridoma cells, and immunofluorescence analysis using fluorescein isothiocyanate (FITC)-conjugated antihuman IgA antibody was performed. The result demonstrated that IgA deposit was efficiently removed by washing with citrate buffer and was recovered after incubation with the culture medium of hybridoma cells [43].

TONSIL STIMULATION AND IgAN

Method and judging criteria of tonsil provocation test

The methods of tonsil provocation test include direct or indirect tonsil stimulation using Tonsil Provocator producing an ultrashort wave (each tonsil for 5 minutes), mechanical tonsil stimulation (tonsil massage, each tonsil for 5 minutes) and injecting hyaluronidase (2000 U/mL, each tonsil for 0.5 mL) into tonsils. In general, four criteria are used to judge the results of tonsil provocation test. Any one of four criteria positive is regarded as tonsil provocation test positive: (*1*) an increase of white blood cell count over 1200/mm^3 after 3 hours; (*2*) an increase in body temperature over 0.55°C after 15 minutes; (*3*) enhancement of erythrocyte sedimentation rate over 12 mm after 1 hour; and (*4*) worsened skin eruption or deterioration of urinary findings after 3 hours, which is defined as urinary protein increased by more than 30 mg/dL or erythrocyte count in the sediment increased by more than 10/hpf, as compared with that before the test [44, 45].

Effect of tonsil stimulation on IgAN

Although the pathogenesis of IgAN still remains uncertain, it is well known that IgAN patients often show gross hematuria or deteriorated urinary findings after upper respiratory tract infections such as tonsillitis, it is supposed that tonsil inflammatory stimulation may be related to IgAN. Masuda et al [46] reported that a tendency of decreasing levels of serum complement combined with an increase of CIC was observed within 1 week after tonsil provocation test in several cases of IgAN associated with chronic tonsillitis [46]. Shiraishi et al [44] performed the tonsil provocation test in 11 cases with pustulosis palmaris et plantaris (PPP) and seven cases with IgAN. Analysis of the provocation test proved positive in three of 11 cases (27%) with PPP and in five of seven cases (71%) with IgAN [44]. Yamabe et al [45] studied effect of ultrashort wave stimulation of tonsils on urinary findings in patients with IgAN. In 62 patients with IgAN and 20 patients with other renal diseases, tonsils were directly stimulated by Tonsil Provocator producing an ultrashort wave to 40.68 MHz each tonsil for 5 minutes. Forty (65%) of 62 patients with IgAN showed deterioration of urinary findings after the stimulation compared with 6 (30%) of 20 patients with other renal diseases. The deterioration of urinary findings was significantly more frequent in IgAN than in other renal diseases. In additional, previous episodes of gross hematuria following upper respiratory tract infections and the level of serum secretory IgA were higher in IgAN patients with deterioration of urinary findings after tonsil stimulation than in those without deterioration [45]. Matsuda et al [47] evaluated the effects of the mechanical tonsil stimulation on the serum and urinary concentrations of macrophage-colony-stimulating factor (M-CSF) in patients with IgAN associated with chronic tonsillitis. The serum and urinary levels of M-CSF in the groups with mild and severe IgAN were significantly higher than those in the chronic tonsillitis group without IgAN. Enhanced urinary excretion of M-CSF prolonged for 7 days after tonsil stimulation in the severe IgAN group; in contrast, the urinary M-CSF