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GLOMERULAR EXPRESSION OF ADHESION MOLECULES AND CYTOKINES IN IgA NEPHROPATHY

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The present study was conducted to assess the expression of adhesion molecules and inflammatory cytokines/growth factors in the renal tissues of IgA nephropathy (IgAN) patients. Renal biopsy specimens from 9 patients and 5 control subjects were stained by the avidin-biotin immunoperoxidase complex (ABC) method using monoclonal antibodies against each of the adhesion molecules and cytokines. Tissue mRNA was detected by the reverse-transcription-polymerase chain reaction (RT-PCR) method to confirm *de novo* synthesis of protein. Increased intercellular adhesion molecule-1 (ICAM-1) staining was observed in the mesangial area in the IgAN specimens. Among the inflammatory cytokines, tumor necrosis factor-α (TNF-α) staining was intense and mRNA expression was increased in the glomeruli, and enhanced staining was correlated with the magnitude of proteinuria. Interleukin-5 (IL-5) expression in the glomeruli was increased in several patients with abundant mRNA, suggesting that IL-5 is involved in the immunoglobulin A modulations in the glomeruli which are prominent in this disease.

Taken all together, adhesion molecules and inflammatory cytokines appear to be aberrantly expressed in the glomeruli of IgAN patients, and analysis of renal biopsy specimens for cytokine expression can provide valuable information for assessing disease activity, clinically.

Introduction

IgA nephropathy (IgAN) is a chronic form of glomerulonephritis characterized histologically by mesangial deposition of immunoglobulin A[1]. However, its clinical manifestations, i.e., disease activity, pathological findings and prognosis vary greatly. In addition, the etiology and pathogenesis of this disease have not been fully clarified.

Several factors are suspected of contributing to the pathogenesis and progression of disease activity in IgAN[2], and it is generally believed that paracrine and/or autocrine stimulation of cytokine and growth factor influences glomerular cell proliferation and expansion of mesangial matrices in patients with IgAN[3][4]. There is increasing evidence that several cytokines secreted by resident glomerular cells and inflammatory cells infiltrating glomeruli up-regulate the expression of cell adhesion molecules and inflammatory cytokines on glomerular cells during the onset and progression of this disease[5]~[9].

It is therefore meaningful to investigate the expression of adhesion molecules and inflammatory cytokines in the glomeruli of patients with IgAN. In the present study, we evaluated their glomerular expression at protein and gene level by immunohistochemical staining and the reverse-transcription-polymerase chain reaction (RT-PCR) amplification method, respec-

tively.

Subjects and Methods

The clinical data and renal biopsy tissues of nine IgAN patients were analyzed. The diagnosis of IgAN was made on the basis of the clinical manifestations and the light microscopic and immunofluorescence findings in the renal biopsy specimens. None of the patients had any clinical or laboratory findings indicative of anaphylactoid purpura, liver disease and systemic diseases. Histologically normal tissue was obtained from the normal part of the kidneys of patients with renal cell carcinoma. Clinical parameters, urinalysis, serum chemistry and renal function were analyzed at the time of biopsy.

Renal biopsy specimens from the IgAN patients were fixed in 10% buffered formalin and embedded in paraffin. Thin-sliced sections were stained with hematoxylin and eosin, by the periodic acid-Schiff method, and with silver methenamine. All glomeruli in each section were examined by light microscopy and histological changes were noted.

Immunohistochemistry

Freshly frozen sections were stained by the avidin-biotin immunoperoxidase complex (ABC) method with commercially available monoclonal antibodies[10]. Briefly, frozen sections were fixed in acetone for 10 min, rinsed with phosphate-buffered saline (PBS, pH 7.2), and incubated with an appropriate blocking agent (Vector Laboratories, Inc., Burlingame, Calif) for 20 min. After incubation for 1 h with primary antibodies, they were incubated with anti-biotinylated rat IgG serum (H + L; Vector Laboratories) as the secondary antibody for 30 min, washed with cold PBS for 30 min and incubated with the ABC reagent (Vector Laboratories) for 30 min. After washing with PBS, the sections were treated with a freshly prepared solution of 0.05% 3,3-diaminobenzidine and 0.005% H_2O_2 in Tris-HCl buffer (0.05 M, pH 7.6) for 5 min, washed with distilled water, and counterstained with methyl green. All controls, which were treated with normal rat serum (Cappel Laboratories, Cochranville, Pa) or PBS instead of the primary antibodies, yielded negative results.

The intensity of staining was graded semiquantitatively as follows: (−) negative, (+) weak staining above background, (++) significantly different from background, (+++) more intense staining.

Monoclonal antibodies

Anti-intercellular adhesion molecule-1 (ICAM-1), anti-vascular cell adhesion molecule-1 (VCAM-1) and anti-endothelial-leukocyte adhesion molecule-1 (ELAM-1) were obtained from British Bio-technology (Oxon, England). The biotinylated rat monoclonal antibodies to human interleukin-1β (IL-1β), interleukin-5 (IL-5), tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) were obtained from Pharmingen (San Diego, Calif).

RNA isolation

To analyze expression of adhesion molecules and cytokine genes by the RT-PCR method, RNA was reverse-transcribed into complementary DNA. Total RNA was prepared from thin-sliced tissue from frozen renal biopsy specimen block, as described previously[11]. Briefly, aliquots of tissues were placed in Eppendorf tubes, and lysed in 200 μl of ice-cold lysis solution D containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, 0.5% (w/v) sarcosyl, and diethyl pyrocarbonate-treated (DEPC) water. This was followed by the addition of 200 μl of phenol, 20 μl of chloroform-isoamyl alcohol (49:1, v/v), and 40 μl of 2 N Na-acetate. The mixtures were chilled on ice for 15 min and centrifuged at 10, 000 g for 20 min at 4°C. The aqueous phase was recovered and mixed with an equal voulme of isopropanol at −20°C for 90 min. After centrifugation at 10,000 g for 20 min at 4°C, solution D was added to the pellets, and incubation was performed at −20°C for 60 min. Following pelleting by centrifugation, the RNA was washed with 75% ethanol in DEPC-treated water and centrifuged at 10,000 g for 10 min at

4°C.

Amplification method

One hundred ng sample of the RNA measured by OD meter was used for RT-PCR amplification. The RNA was reverse-transcribed into complementary DNA. Briefly, after incubating 100 ng of total RNA at 65°C for 5 min, the reaction mixture [20 units of ribonuclease inhibitor, 10× PCR buffer, 1.25 mM dNTPs, 10× hexanucleotide mixture, 0.1% dithiothreitol, reverse-transcriptase (Takara, Tokyo, Japan)] was added to the RNA solution, and the mixture was then incubated at 42°C for 30 min. After incubation, it was heated at 94°C for 5 min to terminate the reaction and then chilled on ice.

The polymerase chain reaction was employed with thin-sliced biopsy samples of mRNA serving as the template and cytokine-specific oligonucleotides as the primers. The reaction was carried out in a 100 μl of solution containing 10 μl of the cDNA solution, 10 mM Tris aminomethane HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1.25 mM dNTPs, 50 pM of the 5′ and 3′ primers, 2 units AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn). The reaction was initiated by denaturing the solutions at 94°C for 5 min, being cycling at 94°C for 1 min (denaturing), 55°C for 1 min (annealing), 72°C for 1 min (extending) for 32 cycles in a Perkin Elmer Cetus Thermal Cycler (Perkin Elmer Cetus, Norwalk, Conn). After the final cycle, the temperature was maintained at 72°C for 10 minutes to allow reannealing of the amplified products, and the mixture was then chilled. The concentrations of primers and polymerase were determined empirically for optimal reaction efficiency.

The specific primer and probes were synthesized by the phosphoramide method using a DNA synthesizer (model 391 PCR-MATE; Applied Biosystems, Inc., Foster City, Calif) and purified on Sephadex G50 columns (Pharmacia LKB Biotechnology, Gaithersburg, Md) and by high-performance liquid chromatography. The sequences of the primers and probes are specific, as confirmed by a computer-assisted search of updated versions of GenBank, and were chosen with balanced nucleotide composition ranging from 40 to 60% of the GC content (Table 1).

A 10 μl product sample was analyzed by electrophoresis on a 1.8% agarose gel, and visualized by ultra violet (UV) fluorescence after staining with ethidium bromide.

Sensitivity and specificity of RT-PCR

The specificity of the amplified product was validated by its predicted size on agarose gel

Table 1 Oligonucleotides used in RNA amplification and hybridization

Product size	Primer	Specific probe
ICAM-1 (351 bp)	5' : TGACCATCTACAGCTTTCCGGC	CTTCTCCTGCTCTGCAACC
	3' : AGCCTGGCACATTGGAGTCTCT	
VCAM-1 (270 bp)	5' : CCAAGAATACAGTTATTTCTGTG	TTCTGGAATTTATGTGTGTGA
	3' : TAGGGAATGCTTGAACAATTAATTC	
ELAM-1 (315 bp)	5' : AGAAGAAGCTTGCCCTATGCT	TTCTTCCTGTCTATCAGGT
	3' : AGGCTGGAATAGGAGCACTCCA	
IL-1β (355 bp)	5' : AAACAGATGAAGTGCTCCTTCCAGG	CACCTTCTTTCCCTTCATC
	3' : TGGAGAACACCACTTGTTGCTCCA	
IL-5 (193 bp)	5' : ATGAGGATGCTTCTGCATTTG	CCACAAGTGCATTGGTG
	3' : CTCATTGGCTATCAGCAG	
TNF-α (325 bp)	5' : CAGAGGGAAGAGTTCCCCAG	CAAACCCTCAAGCTGACG
	3' : CCTTGGTCTGGTAGGAGACG	
INF-γ (270 bp)	5' : AATGCAGGTCATTCAGATG	TGCAGAGCCAAATTGTCTC
	3' : TTGGACATTCAAGTCAGTT	
β-actin (202 bp)	5' : CCTTCCTGGGCATGGAGTCCTG	AAAGACCTGTACGCCAACA
	3' : GGAGCAATGATCTTGATCTTC	

and Southern blot analysis with specific internal probes ^{32}P-end-labeled by the method of Saito et al[12]. Briefly, after denaturing the samples with 1 M sodium hydroxide and neutralizing by addition of 1 M ammonium acetate, the PCR products were transferred to a nylon membrane using with a dot blot apparatus. The blots were hybridized with a γ-^{32}P ATP-end-labeled oligonucleotide probe. Hybridization was allowed to proceed for 3 h at 42°C in a hybridization solution (Formamid, 20× SSC, 0.1 M NaH_2PO_4, pH 7.0, 20% SDS, and nonfat dry milk). The membrane was washed three times in a solution containing 20× SSPE and 20% SDS for 10 min at 40°C, autoradiographed with intensifying screens for 3 h at −70°C, and the film was then developed immediately.

One hundred ng of total RNA from LPS-stimulated peripheral blood monocytes obtained by glass adherence or IL-1β-stimulated HUVEC was diluted 1～5 orders of magnitude with 1 μg of yeast RNA and assayed by RT-PCR to examine the sensitivity of the assay. The amplified product was visualized on a UV illuminator and photographed with type 55 positive/negative film (Polaroid, Cambridge, Ma). The negatives were scanned with a computerized laser densitometer (LKB, Uppsals, Sweden). To quantify relative levels of mRNA, a standard curve was obtained by titration of RNA of LPS-stimulated peripheral blood monocytes or IL-1β-stimulated HUVEC, which had indicated the highest amount of expression (Fig. 1). Relative difference in mRNA expression in the test samples were obtained from the standard curve run the same number of cycles as the unknown samples. The amount of product was expressed as geometric means of OD and were scored on a 4-grade scale: (−), not detectable; (+), (++) or (+++), according to the amount of product detectable.

Statistical analysis

Correlations between the positive immunohistologic staining or signal of PCR amplification and clinical, histological parameters were analyzed by the Wilcoxon-Mann Whitney test, the

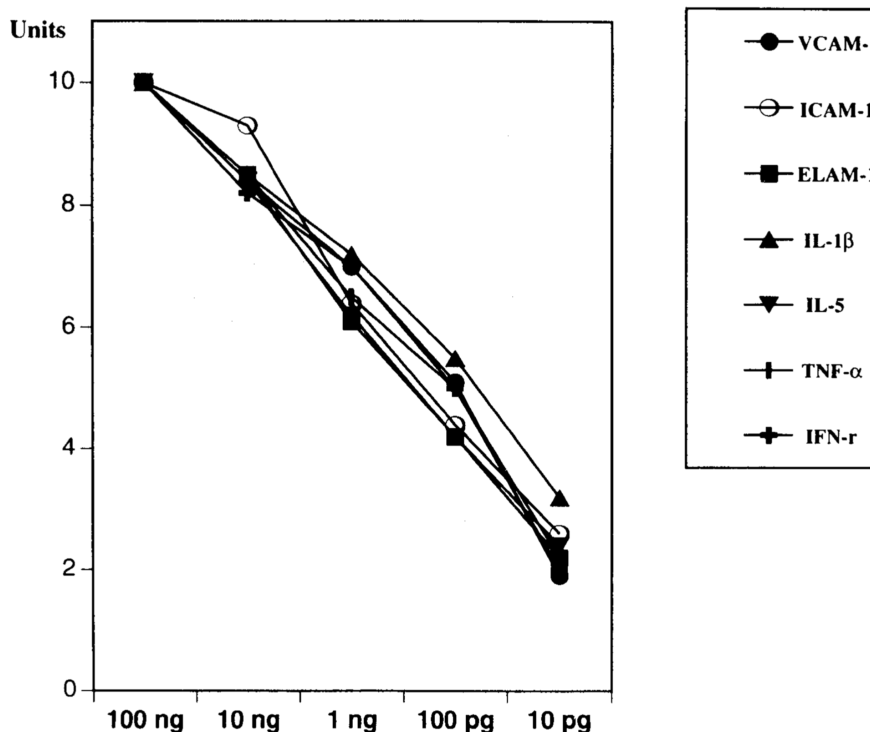

Fig. 1 To quantify relative levels of mRNA, the standard curve was obtained by titration of RNA of Con A-stimulated peripheral blood monocytes. Relative differences in mRNA expression in biopsy specimens were obtained from standard curve run the same number of cycles.

Table 2 Clinical and laboratory profiles of IgAN patients

Case (No.)	Age	Ccr (ml/min)	Urine-protein (g/day)	Urine-RBC (counts/HPF)	Mesangial cell count (counts/glo)
1	36	81.7	1.37	20	37.2
2	18	62.8	2.60	80	33.7
3	37	104.6	0.76	30	36.5
4	25	91.5	0.31	20	30.5
5	34	62.1	0.39	2	37.8
6	37	88.0	0.21	30	35.0
7	32	64.5	3.43	3	32.0
8	21	103.0	0.72	100	30.2
9	42	81.0	1.10	100	38.8

Mesangial cell count: average number of mesangial cells per glomerulus counted in 5 glomeruli.
Ccr: creatinine clearance, RBC: red blood cell, HPF: high-power fields.

χ^2 test and ANOVA.

Results

The clinical profiles and laboratory findings of the IgAN patients have been summarized in Table 2. Creatinine clearnace (Ccr) ranged from 62.1 to 115 ml/min, and there were no patients with chronic renal failure. The average urinary protein level was 1.6 g/day, and 1 patient (case 7) had proteinuria in the nephrotic range.

Immunohistochemical analysis of biopsy specimens

Cryostat frozen sections of all specimens used for PCR analysis were examined by the immunoperoxidase and immunofluorescence techniques. The adhesion molecule VCAM-1 was slightly positive in glomeruli from both normal and IgAN kidneys, but ELAM-1 was negative in the glomeruli. Although ICAM-1 staining was detected in the glomeruli of both control and IgAN patients, the enhancement was observed in the specimens from some, but not all, of the patients (cases 1, 2, 5, 6 and 9) (Fig. 2A, B). It was difficult to differentiate cell types in the glomeruli on frozen sections. Staining intensity was not significantly correlated with clinical manifestations, such as the decrease of Ccr or the magnitude of urinary protein excretion, or with the histological findings.

Inflammatory cytokine staining was more variable. There were no significant differences between IL-1β and IFN-γ staining in the controls and IgAN. IL-5 staining was enhanced in the IgAN glomeruli: (++) in two cases (cases 3 and 9), (+) in five cases (cases 1, 2, 4, 6 and 8); and the others were negative. The IL-5 staining was mainly distributed on endothelial cells and mesangial cells (Fig. 3A, B), but was unrelated to the clinical manifestations and pathological findings. TNF-α staining was also enhanced in the glomeruli in the patients' specimens (Fig. 4 A, B), and the staining was correlated with the level of proteinuria among the clinical parameters ($p<0.05$).

RT-PCR analysis of gene expression

The cDNA transcribed from extracted mRNA was used as a template for the PCR amplification reaction to detect small messages. To confirm the integrity of the tissue RNA, the housekeeping gene "β-actin" was co-amplified with this cDNA and the data were analyzed only when the β-actin signal was detectable. No ELAM-1 signals were amplified in the IgAN or control samples. Although the gene expression of ICAM-1 and VLAM-1 was detected in some specimens from both groups, there were no differences at the amplification levels.

Gene expression of IL-5 and TNF-α was enhanced in the IgAN patients. This was in contrast to the other inflammatory cytokines, for which no significant differences in expres-

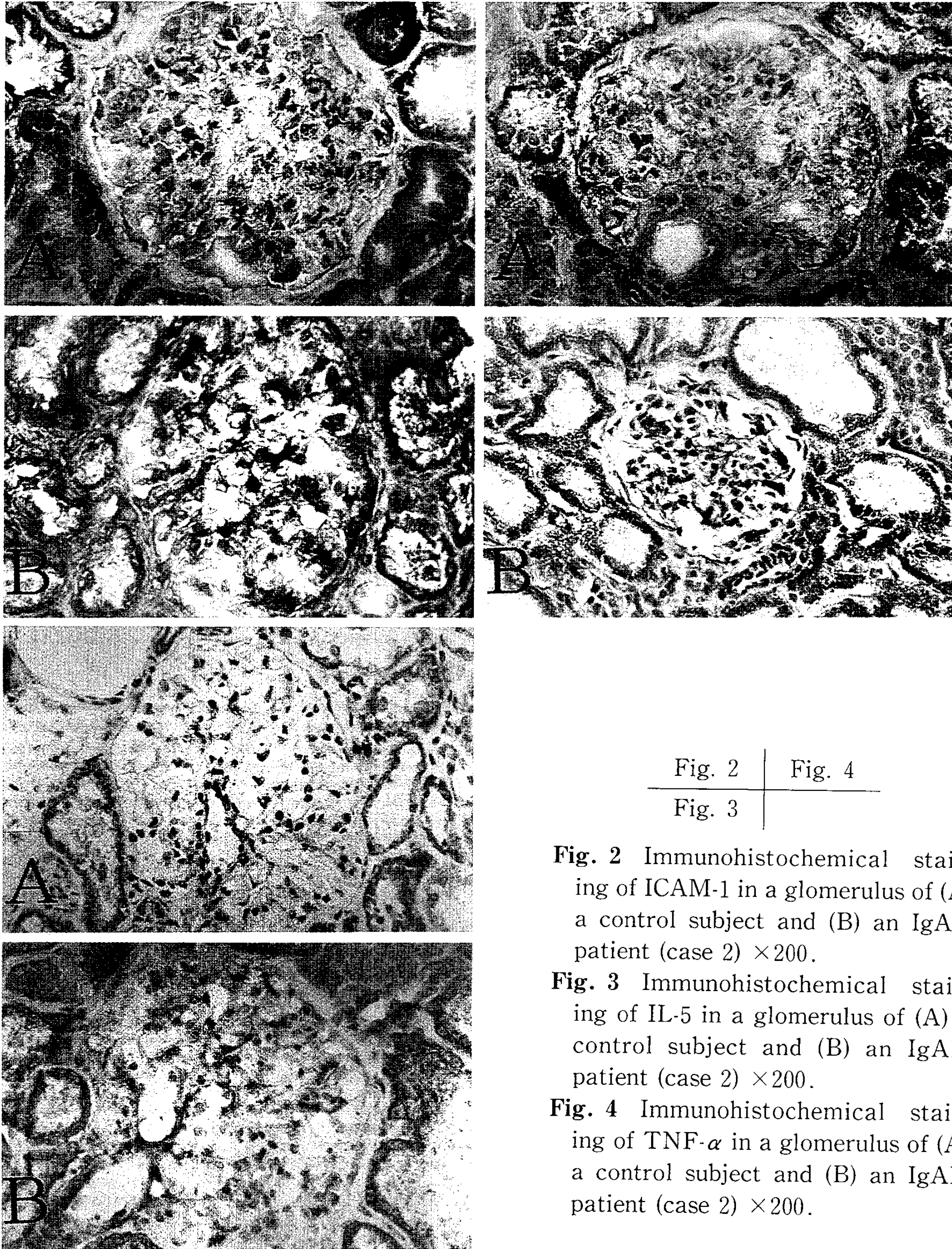

Fig. 2	Fig. 4
Fig. 3	

Fig. 2 Immunohistochemical staining of ICAM-1 in a glomerulus of (A) a control subject and (B) an IgAN patient (case 2) ×200.

Fig. 3 Immunohistochemical staining of IL-5 in a glomerulus of (A) a control subject and (B) an IgAN patient (case 2) ×200.

Fig. 4 Immunohistochemical staining of TNF-α in a glomerulus of (A) a control subject and (B) an IgAN patient (case 2) ×200.

sion levels were detected between the two groups. The amount of PCR product of IL-5 was significantly correlated with one of the histological factors, mesangial cell count ($p<0.05$), to which the intensity of staining was not statistically correlated. Although enhancement of gene expression of TNF-α was observed in the IgAN patients, it was not always related to the magnitude of the proteinuria, in contrast to the immunohistological findings.

Figure 5 shows the representative blotting of the PCR products of these positive cytokines (ICAM-1, IL-5 and TNF-α) in cases 1, 2 and 3, in comparison with three control subjects. Table 3 summarizes the results of intensity of the staining and the magnitude of PCR amplification of ICAM-1, IL-5 and TNF-α, comparing with those of control subjects.

To confirm the specificity of the expression of IL-5 in IgAN, immunostaining and amplification reactions were performed in patiens with other types of glomerulonephritis. The same protocol was carried out in specimens from patients with minimal change disease ($n=4$) and

Table 3 Summary of ICAM-1, IL-5 and TNF-α glomerular immunostaining and gene expression

Case (No.)	ICAM-1		IL-5		TNF-α	
	Staining	PCR	Staining	PCR	Staining	PCR
IgAN						
1	++	+	+	+++	++	+
2	++	++	+	−	+++	++
3	+	+	++	+++	+	+
4	+	++	+	+	++	+
5	++	±	−	−	++	+
6	++	+	+	+	++	+
7	+	+	−	−	+++	++
8	+	−	+	−	++	+
9	++	+	++	++	+	−
Control						
1	+	−	−	−	−	−
2	+	±	−	−	+	−
3	+	+	−	−	−	+
4	−	−	−	−	+	−
5	+	+	+	+	−	−

membranous nephropathy (n=4). No IL-5 signals could be detected in these diseases (data not shown).

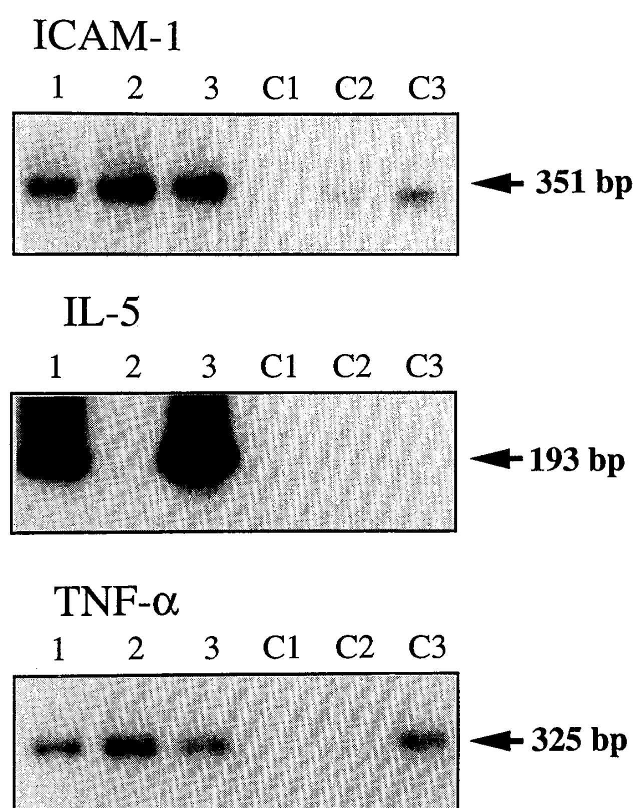

Fig. 5 Detection of cytokine gene expression by RT-PCR
Representative Southern blotting of PCR products hybridized with specific oligonucleotides for ICAM-1 (351 bp), IL-5 (193 bp) and TNF-α (325 bp). Lanes 1, 2 and 3 represent cases 1, 2 and 3, respectively. Lanes C1, C2 and C3 represent control subjects.

Discussion

Dysregulation of cytokine productkion has been implicated in the pathogenesis of IgAN, but the precise mechanism and the role of each component has not been clarified[9].

In this study, we have investigated the expression of adhesion molecule and cytokine in the glomeruli from renal biopsy specimens. Immunohistochemical staining is conventional method for evaluating the expression level of protein. On the contrary, it has been hampered by the limited abundance of specimen and the poor resolution of conventional methods to detect small changes in mRNA levels. Based on recent advances in molecular biology, RT-PCR method enables to amplify small message ex-

pressed in blood, biopsy, and other clinical samples. It is well established that PCR method has improved the sensitivity for detection. There is, however, still controversy on quantitation of the amount of message expressed in the specimen[13]. Although competitive PCR has been reported to be an accurate method for the quantitative analysis[14], we have employed a more conventional method to semiquantitate the message in this study, as the detection of the expression of mRNA is closely relevant to the confirmation of protein expression.

In general, induction of endothelial leukocyte adhesion molecules represents an important initial event in the accumulation of inflammatory cells and expression of cytokines in intraglomerular tissue (i.e., residual cells in the glomeruli) at antigen-stimulated tissue sites. Activation of the inflammatory process may be implicated in the initiation of glomerular damages[15]. Three endothelial cell adhesion molecules have previously been reported to be involved in leukocyte adhesion to endothelium in humans, i.e., VCAM-1, ICAM-1, and ELAM-1[16]. In our study, ICAM-1 staining was more prominently expressed in some IgAN patients, compared to two other adhesion molecules, and this was compatible with the findings in previous reports in various types of glomerulonephritis, renal allografts and immune-mediated renal disease[17]~[20]. Significant changes in renal expression of ICAM-1 have been observed during glomerulonephritis, supporting the hypothesis that this molecule is an important participant in immune interactions, perhaps especially in chronic types of inflammation. The discrepancy in the expression of adhesion molecules could not be explained in this study. The expression of each molecule during the course of the disease might be transient, resulting in uneven detection of their signals.

We found that the protein and gene for the inflammatory cytokines such as IL-5 and TNF-α were enhanced in their expression in the glomeruli. As it is well known that IL-5 induces IgA synthesis, i.e., IL-5 promotes the post-switch differentiation in IgA-bearing B cells[21]. A recent study has shown that IL-5 mRNA is up-regulated by CD4-positive T cells from IgAN patients[22]. In our study, IL-5 was stained positive in glomeruli from IgAN kidneys and IL-5 mRNA was also detectable in them. As to the modulation of IgA production, transforming growth factor-β (TGF-β) is shown to induce IgA subtype switch at a clonal level from the μ to α chain[23] and IL-5 accelerates the post-switch differentiation in IgA bearing B cells. We examined TGF-β staining and gene expression in the glomeruli with same methods, resulting in enhanced immunostaining without detection of gene expression (data not shown). Involvement of TGF-β in sequential process of IgA production has remained uncertain. The significance of IL-5 expression and IgA deposition in the mesangial area has not been clarified. The level of IL-5 gene expression in the glomeruli tended to be correlated with mesangial cell count, suggesting involvement of IL-5 in mesangial proliferative glomerulonephritis, as has been reported for IL-6 previously[24]. Further studies are needed to identify the cells in the glomeruli responsible, for expressing the IL-5 gene and protein.

The staining and mRNA transcript of TNF-α are increased in the glomeruli of IgAN patients, and these findings were found to be correlated with urinary protein excretion, which has been used as a clinical marker of disease activity in this form of glomerulonephritis. Although down-regulation of TNF-α was reported in IgAN patients previously, the report concerned expression in peripheral blood mononuclear cells, in contrast to the renal biopsy tissue in our study[25]. As TNF-α is synthesized by mesangial cells and its synthesis is stimulated by several extrinsic factors[26], it may exert some biologic activity in glomeruli related to the pathologic findings in them. TNF-α is a rapid-turnover cytokine and up-regulation would be expected to be observed in the chronic course of IgAN patients.

In summary, we have demonstrated aberrant

expression of adhesion molecules and inflammatory cytokines in the glomeruli of patients with IgAN. There was enhanced expression of ICAM-1 protein, but not of VCAM-1 and ELAM-1 protein or mRNA, in the glomeruli of some patients. As to the inflammatory cytokines, the staining and gene expression of IL-5 and TNF-α were enhanced in some patients. These findings suggest that dysregulation of the cytokine network is involved in the pathogenesis of the immune abnormalities and tissue damage in this disease[27].

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IgA 腎症における接着因子とサイトカインの糸球体発現

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サイトカインは生体内で複雑なネットワークを形成し，炎症の進展や組織障害，細胞外基質の産生などに重要な役割を演じているとされ，慢性糸球体腎炎でも病因，病態への関与が推測されている．今回，IgA 腎症患者の腎生検組織で接着因子(intercellular adhesion molecule-1: ICAM-1, vascular cell adhesion molecule-1: VCAM-1, endothelial-leukocyte adhesion molecule-1: ELAM-1) および炎症性サイトカイン(interleukin-1β: IL-1β, tumor necrosis factor-α: TNF-α, interferon-γ: INF-γ, interleukin-5: IL-5) の糸球体内発現の検討を行った．

9人の IgA 腎症群の腎生検標本と5人の手術時摘出標本健常部をコントロールとして，各接着因子，サイトカインに対するモノクローナル抗体を用い，染色性の程度を定量した．また mRNA は凍結組織切片より抽出し，reverse-transcription PCR (RT-PCR) 法を用いて検出した．

接着因子では ICAM-1が IgA 腎症の糸球体内での染色性の増加が認められた．炎症性サイトカインでは TNF-α が強く染色され，mRNA の発現の亢進を伴い，その染色性は臨床所見上蛋白尿の程度と相関がみられた．IL-5の糸球体発現が染色性，mRNA ともに亢進した症例が認められ，mRNA はメサンギウム細胞数と有意に相関していた．IL-5は B 細胞の増殖を促進し，IgA 産生の誘導作用が指摘されており，本症の特徴的所見である IgA のメサンギウム領域への沈着のメカニズムに示唆を与えうるものと考えられた．

IgA 腎症の糸球体における接着因子，炎症性サイトカインの発現を免疫組織染色および RT-PCR 法により確認した．各種サイトカインが本症の発症，進展の各時期に関与する可能性が示唆された．