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ORIGINAL ARTICLE

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Detection of enteroviruses in renal biopsies from patients with immunoglobulin A nephropathy

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Abstract Viruses have been suspected to be one of the causes of IgA nephropathy (IgAN). Recent studies have detected viruses in renal tissues of patients with IgAN. Enteroviruses have been reported as pathogenic agents in some renal diseases. We previously reported that group B coxsackieviruses cause pathological changes in experimentally infected mouse kidney. The aim of the present study was to examine the participation of enteroviruses in the pathogenesis of renal diseases including IgAN. Renal biopsies of ten patients with IgAN (group 1) and of 19 patients with non-IgAN renal disease(group 2) were analyzed by polymerase chain reaction (PCR) for the presence of enteroviral RNA. Positive PCR results were obtained for three patients (30%) of group 1. We confirmed by sequencing that the positive PCR products were derived from strains of enteroviruses. One of these three patients also had a positive result for lymphocytes from peripheral blood. In contrast, enteroviral RNA was detected in none of the 19 patients of group 2. The incidence of enteroviral RNA detection in patients of group 1 was higher than that in group 2 ($P<0.05$). Our findings suggest that enteroviral infection may have the possibility of becoming one of the factors involved in the mechanism of onset or evolution of IgAN.

Keywords IgA nephropathy · Enterovirus · Polymerase chain reaction

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Introduction

IgA nephropathy (IgAN) is an idiopathic glomerulonephritis characterized by predominant IgA deposition in the mesangium and is the most common type of glomerulonephritis worldwide. However, its pathogenesis is still unclear. In IgAN, renal signs and symptoms are frequently preceded by episodes of upper respiratory tract infection and/or gastrointestinal infection, suggesting viral infection as the etiology of this disease. Recently, viral antigens [1, 2] were detected in renal tissues of patients with IgAN, and in viral DNA, by polymerase chain reaction or in situ hybridization [3, 4, 5]. Enteroviruses are established or suspected etiological agents in numerous diseases. In particular, group B coxsackieviruses (CBs), which are enteroviruses, have been implicated in several diseases, including pancreatitis, insulin-dependent diabetes mellitus, myocarditis, and myositis [6]. They have also been reported to be etiological agents in some renal diseases [7, 8, 9, 10]. For example, experimental nephritis induced by CB4 was first described by Sun et al. [11], and subsequent reports indicated relationships of CB4 with renal disease in humans [7, 8] and experimental animals [12, 13, 14]. We recently demonstrated glomerular lesions with IgA deposits induced by intraperitoneal inoculations of CB4 in mice [13].

In the present study, renal biopsies and lymphocytes from patients with IgAN or other renal diseases were investigated for the presence of enteroviral genome sequences by polymerase chain reaction (PCR) to determine whether IgAN is an enteroviral-associated disease.

Patients and methods

Patients

From March to December 2001, renal biopsy was performed in 29 patients. Whole-blood samples were collected simultaneously from these patients. The patients were divided into two groups based on histological diagnosis. Group 1 comprised 10 patients diagnosed with IgAN, while group 2 was composed of 19 patients diagnosed with non-IgAN renal disease. The non-IgAN patients consisted of 7

patients with minimal change, 10 with purpura nephritis, and 2 with membrano-proliferative glomerulonephritis. We attempted to detect enteroviral RNA in renal biopsy tissues and lymphocytes from peripheral blood in both groups.

Lymphocyte isolation

Whole blood was collected from 27 of 29 patients into tubes containing anticoagulant (EDTA). The blood was diluted by addition of an equal volume of 0.9% NaCl. The diluted blood was carefully layered over Lymphoprep (Nycomed Pharma AS, Oslo, Norway) in a centrifuge tube and was centrifuged at 800*g* for 20 min at room temperature in a swing-out rotor. After centrifugation, the mononuclear cells formed a distinct band at the sample/medium interface. The cells were removed from the interface. The harvested fraction was diluted with 0.9% NaCl, to reduce the density of the solution, and the cells were pelleted by centrifugation at 250*g* for 10 min.

RNA extraction

Total cellular RNA was extracted from the frozen renal biopsies and peripheral lymphocytes homogenized in a modified guanidine thiocyanate buffer (Isogen LS 750, Wako Pure Chemical Industries, Tokyo, Japan) by an acid phenol–chloroform extraction method.

Detection of enteroviral RNA by PCR

PCR for enteroviral RNA amplification was performed as has been previously described [15, 16]. After RNA extraction, cDNA was synthesized from the extracted RNA. Briefly, a reaction mixture, containing 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 5 mmol/l $MgCl_2$, 0.2 mmol/l each of dNTP, 1 μmol/l of primer F1 (5′-CAAGCACTTCTGTTTCCCCGG-3′) and R1 (5′-ATTGTCACCATAAGCAGCCA-3′), 20 U of ribonuclease inhibitor (Toyobo, Osaka, Japan), 50 U of reverse transcriptase RNaseH Minus (Toyobo), and RNA, isolated from the samples, was prepared. After incubation at 42°C for 60 min, the first PCR mixture, which contained 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 5 mmol/l $MgCl_2$, 0.2 mmol/l each of dNTP, 0.2 μmol/l of primers F1 and R1, and 2.5 U of Taq DNA polymerase (Perkin-Elmer, Norwalk, Conn., USA), was added and the amplification was performed in 40 cycles consisting of denaturation for 1 min at 93°C, primer annealing for 1 min at 45°C, and elongation for 2 min at 72°C.

The second PCR was performed as above, using the second primer pair [primer F2 (5′-TCCTCCGGCCCCTGAATGCG-3′) and R1] and 2 μl of the first PCR product. The nested PCR product was analyzed by electrophoresis in 2% agarose gels. Positive PCR reaction was expected to yield a 155-bp band. Zoll et al. [17] reported that the primer pairs were general primers of 60 different enterovirus serotypes (polioviruses 1 to 3; coxsackieviruses A1 to A24, except A11, A17, A24; coxsackieviruses B1 to B6; echoviruses 1 to 33 except 16, 22, 23).

Sequencing of PCR products

Each PCR product was gel-purified and sub-cloned into pGEM-T vector (Promega, Madison, Wis., USA). The sequences of the cloned genes were determined by an automated DNA sequencer (Perkin-Elmer) and screened for homology with database sequences using the BLAST search algorithm.

Pathological examination

The renal biopsy specimens were assessed by light microscopy (LM) and immunofluorescence (IF) examination. Material for histological study was fixed in 20% neutral formalin and embedded in paraffin, sliced at 2–3 μm thickness and stained with hematoxylin and eosin or periodic acid–Schiff reagent.

Tissue samples for IF were immediately fixed in ornithine carbamoyltransferase (OCT) compound and frozen at −80°C until required. IF examination was performed for IgG, IgA, IgM, C1q, C3, C4, and fibrinogen. We used fluorescein-conjugated goat antibodies (IgG) to rabbit IgG, IgA, IgM, C1q, C3, C4, and fibrinogen. The findings and classification of glomerular abnormalities are listed in Table 1. Classification was based on the second edition of the histological classification of glomerular diseases by a committee of the World Health Organization. [18]. The intensity of immunofluorescence was graded as (−) = negative, (+) = mild, (++) = moderate and (+++) = strong.

Definitions and diagnostic criteria

Hematuria was considered present if microscopic examination revealed five or more red blood cells per high-power field, and macro-hematuria if blood was visible with the naked eye [19]. Proteinuria was evaluated by 24-h quantitative measurement.

Diagnosis of IgAN was based on the presence of IgA as the sole or predominant Ig in the glomerular mesangium without systemic disease such as Henoch–Schönlein purpura (HSP) or systemic lupus erythematosus. Diagnosis of HSP was made if the major manifestations of disease were purpuric rash and abdominal pain without thrombocytopenia. Additional features, including arthritis and nephritis, were accepted as consistent with the diagnosis. Membranoproliferative glomerulonephritis (MPGN) was diagnosed by light microscopy based on findings of enlarged and lobular glomeruli, an increase in mesangial cells and matrix, and double contour of the capillary loop. In this study, MPGN was considered a primary disease itself, apart from systemic diseases such as monoclonal nephropathy, anti-phospholipid antibody syndrome, lupus nephritis, other autoimmune diseases, and hepatitis B or C viral infection.

The clinical status of each patient at the most recent observation was classified as follows: *Normal* the patient was healthy on physical examination, with normal urinalysis results and normal renal function. *Minor urinary abnormalities* the patient was healthy on physical examination, with microscopic hematuria or proteinuria less than 40 mg/m^2 per hour. *Active renal disease* the patient had proteinuria of 40 mg/m^2 per hour, or greater, or hypertension, and a 24-h creatinine clearance (Ccr) of 60 ml/min per 1.73 m^2 or greater. *Renal failure* the patient had a 24-h Ccr of less than 60 ml/min per 1.73 m^2 (including cases of dialysis/transplantation or death).

Acute exacerbation was defined as exacerbation of the clinical condition by at least one status level.

Statistics

Values are means ± SEM. Statistical analysis was performed on a Macintosh computer with a software package for statistical analysis (StatView, Abacus Concepts, Berkeley, Calif., USA). Differences among group values were assessed by the Mann–Whitney rank-sum test. Fisher's test was used to evaluate correlations. Findings of $P<0.05$ were considered significant.

Results

Comparison of baseline characteristics between groups

The baseline characteristics of the groups are shown in Tables 1 and 2. Age at onset, and mean duration of follow-up, were 11.2±3.2 years and 5.5±3.9 years, respectively, in group 1 and 8.4±3.5 years and 6.8±3.8 years, respectively, in group 2. Male-to-female ratios were 4/6 and 5/14, respectively.

Table 1 Histopathological findings in each group. Grading of pathological alternations: − to +++. (*D* diffuse, *S* segmental, *F* focal, *G* global, *M* mesangial, *HSPN* Henoch–Schoenlein purpura nephritis, *MC* Minimal change, *C* capillary, *NT* not tested)

Case no.	Age (years)/ gender	Diagnosis	Light microscopy			IF staining							PCR results		Clinical status	Acute exacerbation
			Mesangial lesion		Interstitial cell infiltration	IgG	IgA	IgM	C1	C3	C4	Fibrinogen	Renal biopsies	Lympho-cytes		
			Cell prolifer-ation	Matrix expan-sion												
Group 1																
1	13/Male	IgAN	D,S	D,S	+	M++	M+++	M+	−	M+	−	MC+	−	NT	Unknown	Unknown
2	14/Male	IgAN	D,S	-	+	M+	MC+++	M+	−	M+	−	−	−	-	Minor	-
3	8/Female	IgAN	F,S	F,S	−	−	MC++	−	−	−	−	C++	−	−	Unknown	Unknown
4	9/Female	IgAN	D,S	D,S	+	−	M+++	M++	−	−	−	MC++	−	−	Minor	−
5	16/Female	IgAN	F,S	F,S	−	M++	M+++	M+	−	M++	−	C+	−	−	Active	+
6	6/Female	IgAN	F,S	F,S	−	M++	M+++	M+	−	−	−	M+++	+	−	Minor	−
7	19/Male	IgAN	F, S	F, S	+	−	M++	M+	−	M++	−	−	+	+	Minor	−
8	14/Female	IgAN	F,S	F,S	+	−	M+++	−	−	M++	−	MC+	−	−	Minor	−
9	14/Male	IgAN	F,S	F,S	−	MC+	M+++	−	−	M+	−	M++	+	NT	Minor	−
10	9/Female	IgAN	D,S	–	+	−	MC++	MC+	−	MC+	−	C+	−	−	Normal	−
Group 2																
11	8/Male	HSPN	F,S	F,S	−	−	M+++	M+ −	−	M+	M+	M++	−	-	Unknown	Unknown
12	13/Female	MC	–	–	+	−	−	−	C++	−	−	M++	−	−	Normal	−
13	9/Male	MC	–	–	+	−	−	−	−	−	−	−	−	−	Minor	−
14	17/Male	MC	–	–	+	−	−	−	−	C++	−	−	−	−	Minor	−
15	11/Female	MC	–	–	−	−	C++	−	−	−	−	−	−	−	Minor	−
16	9/Female	HSPN	–	F,S	−	−	MC++	MC+	−	C+	−	C+	−	−	Active	+
17	11/Female	HSPN	F,G	F,G	−	−	MC++	−	−	MC+	−	MC+	−	−	Active	−
18	8/Female	HSPN	D,G	D;G	Fibrosis	−	M+++	−	−	C+	−	C++	−	−	Active	−
19	14/Female	HSPN	D,S	D,S	+	−	MC+++	MC+	−	MC++	−	MC++	−	−	Active	−
20	13/Female	MPGN	D,G	D,G	−	C++	−	−	C++	C+++	−	C+	−	–	Unknown	Unknown
21	5/Male	HSPN	D,S	–	−	−	MC++	C+	−	MC++	−	MC+	−	−	Normal	−
22	13/Female	MC	–	–	−	−	−	C+	−	−	−	C+	−	−	Normal	−
23	11/Female	MPGN	D,G	D,G	−	−	−	−	−	C+++	−	MC++	−	−	Unknown	Unknown
24	9/Female	HSPN	F,S	–	+	−	M+++	−	−	MC++	−	MC+++	−	−	Minor	−
25	11/Female	MC	–	–	−	−	−	−	−	−	−	−	−	−	Minor	−
26	8/Female	HSPN	D,G	–	+	MC+	MC+++	−	−	C+	C+	MC+++	–	–	Active	–
27	13/Female	HSPN	F,S	–	+	−	MC+++	−	−	−	−	C++	−	Minor	Minor	–
28	4/Male	MC	–	–	−	−	−	C+	–	–	–	−	−	−	Normal	−
29	7/Female	HSPN	F,S	–	+	−	MC+++	−	–	MC++	−	MC+++	−	Minor	Minor	–

Table 2 Comparison of baseline characteristics between both groups

Characteristic		Group 1	Group 2
Age at onset	(years)	11.2±3.2*	8.4±3.5*
Mean follow-up	(years)	5.5±3.9	6.8±3.8
Male:female		4:6	5:14
Mean urinary protein excretion	(g/day)	0.5±0.5	1.8±2.7
Hematuria	(case %)	10 (100%)	14 (73%)
Serum creatinine	(mg/dl)	0.6±0.2	0.7±0.5
Serum total protein	(g/dI)	6.6±0.3	6.5±1.2
24 h Ccr	(ml/min per 1.73 m^2)	145.6±30.1	103.1±51.7

*$P<0.05$

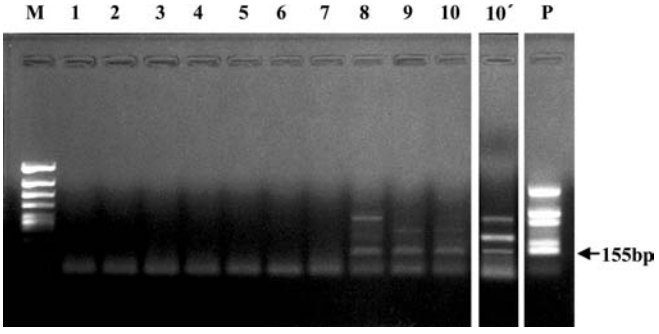

Fig. 1 Gel electrophoresis of PCR products of enteroviral amplification. *Lane M* length marker. *Lanes 1 to 10* are renal biopsies: *lane 1* case 1, *lane 2* case 2, *lane 3* case 3, *lane 4* case 4, *lane 5* case 5, *lane 6* case 8, *lane 7* case 10, *lane 8 case 9*, *lane 9* case 6, *lane 10* case 7. *Lane p* is the positive control (echovirus 7 cDNA clone). *Lane 10'* is case 7 lymphocytes

Mean urinary protein excretion ranged from 0.05 g/day to 1.5 g/day (mean 0.5±0.5 g/day) in group 1 and 0.1 g/day to 10.0 g/day (mean 1.8±2.7 g/day) in group 2. Hematuria was found in 100% of patients in group 1 and in 73% of patients in group 2. Serum creatinine, total protein, and 24-h Ccr concentrations were similar in the two groups.

Detection of enteroviral RNA

Detection of enteroviral RNA is illustrated in Figs. 1 and 2). Positive PCR results were found in three of ten (30%) frozen renal biopsies from the patients of group 1 (Figure 1). One of these three patients also had a positive result for lymphocytes obtained from peripheral blood. In contrast, enteroviral RNA was detected in none of the 19 frozen renal biopsies from patients in group 2. The incidence of enteroviral detection was higher in group 1 than in group 2 ($P<0.05$). To confirm that the PCR products represented enteroviral genome sequences, the sequences of the cloned genes from renal biopsy specimens of cases 6 and 7 were screened for homology with a database using the BLAST search algorithm (Fig. 2). The sequences of the PCR products from cases 6 and 7 were most strongly homologous with a part of human coxsackievirus B1. However, the sequence deviated from that of human coxsackie B1 virus at one base in case 7, and at three bases in case 6. In group 1, none of the clinical manifestations, the degree of exacerbation after onset, pathological findings, and prognosis differed between patients with and without detection of enteroviral RNA (Table 1).

Discussion

There have been many reports on the relationship between viral infection and renal injury [1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14]. Clinically, it has been noted that viral infection often provokes transient proteinuria, hematuria, and renal dysfunction in patients who develop IgAN. Although IgAN is a very common form of glomerular disease, its pathogenesis has yet to be elucidated. Previous studies have revealed that human cytomegalovirus (HCMV) participated in the pathogenesis of IgAN [2, 3]. Recently, Iwama et al. [5] reported that Epstein–Barr

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                  Case 6  *---------  ----------  ----------  ----------  ----------  ----------
                  Case 7  ----------  ----------  ----------  ----------  ----------  ----------
                        446bp                                                            505bp
Human coxsackievirus B1  ggctaatcct  aactgcggag  caggcaatca  caatccagtg  ggtggcctgt  cgtaacgggc
     Human echovirus 26  ***-------  ----------  ------cc--  ---g------  --ca------  ----------
   Human enterovirus 71  ----------  ----------  ----tgcc--  ---c------  ----------  ----------
          Echovirus 6    --------t-  ----------  ----tgct--  ----------  ----------  ----------


                  Case 6  --a-------  ----------  ---------*  -----*----  ----------  ------
                  Case 7  ----------  ----------  ----------  ------*---  ----------  ------
                        506bp                                                            561bp
Human coxsackievirus B1  aactccgcag  cggaaccgac  tactttgggt  gtccgtgttt  ctttttattc  ttacat
     Human echovirus 26  -----t----  ----------  ----------  ------g---  ----------  -----
   Human enterovirus 71  -----t----  ----------  ----------  ------g---  -c--------  --
          Echovirus 6    -----t----  ----------  ----------  ------
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Fig. 2 Comparison of sequences of PCR products and RNA sequences of enteroviruses

virus (EBV)-specific DNA in renal biopsies was detected by PCR in seven (58%) of 12 patients with IgAN, three (50%) of six patients with membranous nephropathy, none (0%) of ten patients with minor glomerular abnormalities, and two (100%) of two patients with focal segmental lesions. EBV genome detection did not yield findings specific for IgAN.

Enteroviruses have been implicated in a wide variety of clinical disorders [6] and have been suggested to be pathogenic agents in renal diseases such as hemolytic uremic syndrome (HUS) and glomerulonephritis [9, 10]. By using pure cultures of human glomerular and tubular cells, Conaldi et al. demonstrated that the infection of six CBs led to cytolysis in proximal tubular epithelial cells and glomerular podocytes [20]. Previously, we reported that CB4 caused pathological changes in experimentally infected mouse kidney [12, 13, 14]. We recently observed lesions similar to those of human IgA nephropathy in mice intravenously inoculated with CB4 once a month from 1 to 5 months of age, and we detected CB4 viral RNA in the mesangial lesions, using in situ hybridization [13]. We therefore attempted to detect enteroviral RNA in renal biopsies from patients with IgAN, using PCR. The PCR method we used could detect almost all prevalent enteroviruses in Japan, with a sensitivity a thousand times that of the virus isolation method [15].

In the present study, we detected enteroviral RNA in frozen renal biopsies from three (30%) of ten patients with IgAN, one of whom also had an enteroviral genome in peripheral lymphocytes. In contrast, enteroviral RNA was detected in none of the frozen renal biopsies from patients with non-IgAN renal disease. Our findings suggest that the detection of enteroviral RNA in renal tissue may be specific for IgAN. No specific findings are available regarding the ability of enterovirus persistently to infect human renal tissue in vivo. However, it has become apparent that CBs are associated with persistent infections in vitro [21, 22, 23]. It thus appears possible that persistent enteroviral infection might be involved in the pathogenesis of IgAN.

In conclusion, our findings suggest that enteroviral infection may have the possibility of becoming one of the factors involved in the mechanism of onset or evolution of IgAN. However, further studies will be needed to determine whether enteroviral RNA can be detected in renal biopsies in other patient populations and whether enteroviral infection plays an important role in the pathogenesis of IgAN.

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