Complications of IgA Nephropathy in a Non-Insulin-Dependent Diabetes Model, the Akita Mouse

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HASEYAMA, T., FUJITA, T., HIRASAWA, F., TSUKADA, M., WAKUI, H., KOMATSUDA, A., OHTANI, H., MIURA, A.B., IMAI, H. and KOIZUMI, A. *Complications of IgA Nephropathy in a Non-Insulin-Dependent Diabetes Model, the Akita Mouse*. Tohoku J. Exp. Med., 2002, **198** (4), 233–244 — The Akita mouse, which has a mutation (Cys96Tyr) in the insulin 2 gene (*Ins2^{Akita}*), is a model for diabetes. The male Akita mouse has diabetes, while females develop mild diabetes. This study aimed to investigate renal complications in the Akita mouse model, which has been maintained in a heterozygous state *Ins2^{Akita}* (+/–) with a C57BL/6 background (B6). Renal function (BUN, creatinine), serum IgA concentrations and histological changes in the kidneys were evaluated in diabetic and control mice in a B6 background. Five each of male and female mice per group (diabetes vs. control) were killed at 10, 20, 30 and 40 weeks of age. The influence of major histocompatibility antigens (MHC) on renal complications was tested using male diabetic mice in a C3H/He (C3H) background. When compared with controls, diabetic males, but not females, developed impaired renal function with elevation of serum IgA after 30 weeks of age. Diabetic glomerulosclerosis advanced with age in both sexes. Diffuse granular mesangial deposits of IgA were detected by immunofluorescence microscopy in diabetic males after 20 weeks. We tested whether the IgA-associated lesions were influenced by MHC using diabetic males in a C3H background. Similar degrees of diabetic glomerulosclerosis and glomerular IgA deposition were found in mice with C3H and B6 backgrounds. Akita mouse is a unique mouse model showing both mesangial sclerosis and IgA nephropathy. — C57BL/6 Akita mouse; diabetic glomerulosclerosis; glomerular IgA deposition; MHC
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The most serious complication of diabetes mellitus is diabetic nephropathy, which is characterized pathologically by glomerulosclerosis, glomerular hypertrophy and deposition of IgG (Tomino et al. 1988; Inoue 1989) and physiologically by albuminuria (Mogensen 1999; Ritz 1999). Recently, we established a mouse model for non-obese type 2 diabetes, the Akita mouse, characterized by an early age of onset and an autosomal dominant mode of inheritance (Yoshioka et al. 1997; Kayo and Koizumi 1998). The gene locus, located on the distal end of chromosome 7, was previously called *Mody 4*. Mutation analysis revealed 1907 G to A transition (Cys96Tyr) in the insulin 2 gene in the Akita mouse (*Ins2^{Akita}*). This mutation replaces the 7th cysteine residue in the insulin chain with tyrosine, and was shown to result in β -cell dysfunction through impaired proinsulin processing (Wang et al. 1999).

The Akita mouse (*Ins2^{Akita} +/−*) (hereafter simply referred to as diabetic), which shows a diabetic phenotype in the heterozygous state, appears a good model to investigate pathological consequences of diabetic nephropathy for the following reasons. First, the involvement of a single gene can eliminate the possible involvement of complex genetic traits for diabetic nephropathy. Second, the blood glucose levels in the male diabetic mouse exceed 500 mg/100 ml (27.8 mM) from 8 weeks of age while female diabetic mice develop only mild diabetes (Yoshioka et al. 1997). The goal of the present study was to investigate the natural course of renal complications in the Akita mouse utilizing physiological and morphological methods.

MATERIALS AND METHODS

Experimental design

In the first set of experiments, we evaluated the time course of diabetic nephropathy in B6 mice. Offspring of diabetic (*Ins2^{Akita} +/−*) male and control female mice (*Ins2^{Akita} −/−*) were genotyped at 8 weeks of age (Wang et al.

1999). Male and female mice of each genotype (5 mice per group, 20 mice in total per age group) were killed at 10, 20, 30 and 40 weeks of age. Renal function was evaluated, and biochemical analysis and histopathological examinations performed.

In the second set of experiments, because major histocompatibility antigens (MHC) are known to markedly influence the development of IgA nephropathy (Berthouix et al. 1978; Brettle et al. 1978; Sabatier 1979; Li et al. 1991; Ibels and Gyory 1994; Fennesey et al. 1996), we evaluated genetic influences on the biochemical profile and renal lesions in the diabetic mice. For this purpose, we used the *Ins2^{Akita} (+/−)* congenic mouse line in a C3H background. Only male diabetic and control mice were used because both renal lesions and diabetes are more marked in males than in females. These mice were killed and evaluated functionally and pathologically similarly to the B6 mice.

In a separate experiment, morning blood glucose was monitored monthly under conditions of ad libitum feeding in different groups of mice from 8 to 40 weeks of age.

Animals

The Akita mouse (*Ins2^{Akita} +/−*), originally derived from the B6 strain, has been maintained as heterozygotes in a B6 background. Animals were genotyped at 8 weeks of age. A blood sample (20 μ l) was collected from the tail vein of each mouse and DNA extracted using a Genomic DNA Purification Kit (Promega, Madison, WI, USA). Mice were assigned to diabetes or control (control) groups after genotyping by PCR using a forward primer (5'-TGC TGA TGC CCT GGC CTG CT-3') and a reverse primer (5'-TGG TCC CAG TAT TGC ACA TG-3'). The primers were designed to amplify exon 3 of the insulin 2 gene on chromosome 7 (Wang et al. 1999) with a product size of 280 bp. The wild-type allele (*Ins2*) was cut into two 140 bp fragments by

Fnu4HI, while in the mutant allele (*Ins2^{Akita}*) the Fnu4HI (Takara, Tokyo) site is disrupted and thus the mutant remained undigested.

Male diabetic (*Ins2^{Akita}* +/−) mice with a C3H background were obtained by repetitive backcrossing of male diabetic mice with control C3H females for more than 10 generations. Mice of these two backgrounds were maintained under specific pathogen-free conditions until sacrificed. Mice were fed commercially available lab chow (CE-2 [Japan Clea, Tokyo] diet [%W/%W]: protein 24.8%, fat 4.4%, carbohydrate 51.6%, and energy 1445 J/100 g).

In a separate experiment, we monitored blood glucose levels from 8 to 40 weeks of age in cohort mice. These mice were composed of diabetic or control mice of each sex with a B6 background, and diabetic and control males with C3H backgrounds. Each group was composed of 20 mice. Blood glucose was determined under ad libitum feeding conditions using a Tidex monitor (Miles-Sankyo, Tokyo) between 9:00 and 11:00 a.m. by sampling from tail veins, as reported previously (Yoshioka et al. 1997).

All mice were handled in accordance with the Animal Welfare Guidelines of Akita University (1990).

Biochemical study

On the day of sacrifice, body weights were measured and mice were transferred to small metabolic cages and housed individually. Urine excreted from each animal was collected for 4 hours from 06:00 a.m. to 10:00, after which they were killed. During the collection period, mice were given free access to water without food. At autopsy, kidney weights were measured and blood was collected by cardiac puncture. Serum samples were separated from the whole blood, and both serum and urine samples were frozen at −80°C until use.

Concentrations of blood urea nitrogen (BUN), creatinine in urine and serum, albumin in serum, and IgA in serum were determined.

We calculated creatinine clearance standardized by kidney weight. Serum IgA concentration was measured by single-radial immunodiffusion analysis after reduction with dithiothreitol and alkylation with iodoacetamide.

Pathological analysis

The left kidney was excised, and immediately cut into four pieces: one piece was fixed in 10% neutral phosphate-buffer formalin for hematoxylin eosin staining and another in phosphate-buffer saline ethanol for PAS staining. The third was immediately frozen in OTC compound (Sakura Finetechnical Co., Ltd., Tokyo) at −80°C for immunohistochemistry and the fourth fixed in glutaraldehyde for electron microscopy.

We evaluated about 100–200 glomeruli in specimens from each animal, and the severity of glomerular changes was graded on a scale of 0 through IV according to Gellman's criteria (Gellman et al. 1959; Takazakura et al. 1975). Briefly, we graded the most advanced changes as the grade of glomerulosclerosis for a given renal specimen: Grade 0; normal glomerulus: Grade I; lesion local within each glomerulus and focal within the kidney: Grade II; diffuse mesangial thickening within the glomerulus and generalized through the kidney: Grade III; capillary lumens narrowed and only locally obliterated: Grade IV; capillary lumen generally narrowed and the whole glomerulus shows ischemic and hyalinized changes.

Sections for immunohistochemistry were cut with a cryostat and subsequently stained with fluorescently labelled anti-sera specific for mouse IgG, IgA or IgM (Kirkegaard and Perry Lab., Inc., Sun city, CA, USA) or C3 (Dako, Glostrup, Denmark). We prepared one section per animal for each immunoglobulin and evaluated the intensities of depositions of each substance. Approximately equally sized pieces of the left kidney were taken from five mice from each group, except from the 40-week old C3H male diabetic group, which comprised just

4 mice. We evaluate all glomeruli in individual specimens, and graded them on the following scale: 0=negative (point 0), I=trace (point 1), II=mild deposition (point 2), III=heavy or intensive deposition (point 3), after photographing under a universal fluorescence microscope (Zeiss, Oberkochen, Germany). The most prevalent grade of deposition of each component in glomeruli was considered to represent the grade of a given mouse for a given component.

Statistical analysis

Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC, USA), we employed parametric and nonparametric statistical methods; nonparametric methods (Mann-Whitney's U-test or Kruskal-Wallis rank test) were used to compare grades among age groups or between diabetic and control groups.

Continuous values were presented as

means $\pm$ s.d. and were compared by analysis of variance (ANOVA); except for serum IgA concentrations, which were compared by parametric and nonparametric methods using both ANOVA and Mann-Whitney's U-test because some values exceeded the limit of detection i.e., 550 mg/100 ml. We treated saturated values as 550 mg/100 ml for statistical analysis. A *p*-value of less than or equal to 0.05 was considered significant. When two statistical tests were applied, we assumed statistical significance only when *p*-values obtained by both methods were significant.

RESULTS

Diabetic phenotypes

After 8 to 40 weeks of age, the mean morning blood glucose level under ad libitum feeding conditions in the diabetic males was 491 ± 95 mg/100 ml ($n=20$) for mice in the B6 back-

Fig. 1. Time course of serum BUN, Creatinine (Cr) and creatinine clearance standardized by kidney weight (CCr). Means and standard deviations (bars) for B6 males and females, and C3H male mice are shown. Results for five mice per group for each sex were averaged. Symbols represent male B6 diabetic mice (■) and controls (□), female B6 diabetic mice (●) and controls (○), male C3H diabetic mice (◆) and controls (◇). **p* < 0.05, ***p* < 0.01 by ANOVA.

ground and 470 ± 75 ($n=20$) for those in the C3H background. For control males, these levels were 167 ± 21.6 ($n=20$) for the B6 background and 123 ± 25 ($n=20$) for the C3H. Although the blood glucose levels tended to be lower in either diabetic or control mice with C3H background than with B6, the difference was not biologically significant.

The mean morning blood glucose levels under ad libitum feeding conditions were 245 ± 35 mg/100 ml for diabetic females and 156 ± 20 mg/100 ml for control females.

Time course of changes in renal function in B6 mice

The BUN levels began to increase at 30 weeks of age in male diabetic mice and were significantly elevated at 40 weeks of age: 24.4 ± 4.5 (mg/100 ml; $n=5$) for control mice and

45.7 ± 14.5 (mg/100 ml; $n=5$) for diabetic mice (Fig. 1). In contrast, those in female diabetic and control mice did not differ significantly.

The creatinine clearance standardized by renal weight (Ccr) was significantly lower after 20 weeks of age in diabetic males (Fig. 1). However, the decrease was not accompanied by changes in serum creatinine levels. For females, there were no significant differences in Ccr between diabetic and control mice.

The serum albumin levels were significantly lower after 30 weeks of age in male diabetic mice (Fig. 2), while those in female mice were not significantly different at any age.

Serum IgA concentrations were elevated significantly in diabetic male mice after 30 weeks of age (Fig. 3). In contrast, there were no significant differences in these values for diabetic or control females.

Fig. 2. Time course of changes in serum albumin. Means and standard deviations (bars) of B6 males and females are shown. Five mice per group for each gender are averaged. Symbols represent male B6 diabetic mice (■) and controls (□), female B6 diabetic mice (●) and controls (○). * $p < 0.05$, ** $p < 0.01$ by ANOVA.

Fig. 3. Time course of changes in serum IgA levels. Means and standard deviations (bars) of B6 males and females, and male C3H mice are shown. Five mice per group for each gender were averaged. IgA concentrations were compared by parametric and nonparametric methods using both ANOVA and the Mann-Whitney's U-test (see to METHODS section for details). Saturated values were treated as 550 mg/100 ml in statistical tests. Symbols represent male B6 diabetic mice (■) and controls (□), B6 female diabetic mice (●) and controls (○), male C3H diabetic male mice (◆) and controls (◇). * $p < 0.05$; ** $p < 0.01$ by ANOVA and Mann-Whitney's U-test.

Pathological study in B6

Hydronephrosis, characterized by expansion of the renal pelvis, was observed in all diabetic males ($n=5$ for each age group) at 30 and 40 weeks of age, but in none of the male control mice, nor in either diabetic or control females (data not shown).

Grades of renal lesions progressed significantly in diabetic males (Figs. 4A-4C). In contrast, renal lesions were significantly less extensive in female diabetic mice (Fig. 4D). Such changes were not found at 40 weeks of age in control males (Fig. 4E). The grades of glomerular lesions for each age group are summarized in Table 1.

Fig. 4. Progression of glomerular lesions in diabetic mice with B6 background.
 A: Normal glomerulus in a male control mouse. This mouse was autopsied at 10 weeks of age. (PAS staining, $\times 400$)
 B: Grade II diffuse diabetic glomerulosclerosis in a diabetic B6 mouse at 20 weeks of age. The lesions are located within each glomerulus and are focal within the kidney. (PAS staining, $\times 400$)
 C: Grade III diffuse diabetic glomerulosclerosis in a diabetic male B6 mouse at 40 weeks of age. Marked mesangial expansion with narrowing of capillary lumen. Small nodular like lesions (arrows) and Armani-Epstein bodies can be seen (arrowhead). (PAS staining, $\times 400$)
 D: Grade II diffuse diabetic glomerulosclerosis in a female diabetic B6 mouse at 40 weeks of age. Mild to moderate mesangial expansion can be seen. (PAS stain, $\times 400$)
 E: Normal glomerulus in a male control B6 mouse at 40 weeks of age.

Glycogen deposits (Armani-Epstein body) were detected in proximal tubuli in diabetic male mice (Fig. 4C, arrowheads). This change, which was detected only in male diabetic mice, was found after 20 weeks of age.

Immunofluorescence microscopy indicted strong diffuse granular mesangial deposits of IgA, in all diabetic males after 20 weeks of

(Figs. 5A and 5B). The grades of IgA deposits increased with age only in diabetic males (Table 2, Kruskal-Wallis rank test). No deposits of IgG, IgM or of C3 were detected in diabetic mice even by intensive immunohistochemical analysis (data not shown). Although IgA deposits were found in control male mice, they were much less extensive than those in diabetic males

TABLE 1. *Light microscopic examination of progression of the grade of glomerular lesions in diabetic and control B6 and C3H mice at different ages*

Diabetic						Control					
B6 male		Grade (No of mice)				B6 male		Grade (No of mice)			
Age (Weeks)	0	I	II	III	IV	Age (Weeks)	0	I	II	III	IV
10	5	0	0	0	0	10	5	0	0	0	0
20	0	1	4	0	0	20	5	0	0	0	0
30	0	0	1	4	0	30	5	0	0	0	0
40	0	0	2	3	0	40	5	0	0	0	0
Kruskal-Wallis rank test $p < 0.05$.						Kruskal-Wallis rank test $p > 0.05$.					
B6 female		Grade (No of mice)				B6 female		Grade (No of mice)			
Age (Weeks)	0	I	II	III	IV	Age (Weeks)	0	I	II	III	IV
10	5	0	0	0	0	10	5	0	0	0	0
20	2	1	2	0	0	20	5	0	0	0	0
30	1	3	1	0	0	30	5	0	0	0	0
40	0	3	2	0	0	40	5	0	0	0	0
Kruskal-Wallis rank test $p < 0.05$.						Kruskal-Wallis rank test $p > 0.05$.					
C3H male		Grade (No of mice)				C3H male		Grade (No of mice)			
Age (Weeks)	0	I	II	III	IV	Age (Weeks)	0	I	II	III	IV
10	5	0	0	0	0	10	5	0	0	0	0
20	2	2	1	0	0	20	5	0	0	0	0
30	0	1	2	2	0	30	5	0	0	0	0
40	0	0	2	3	0	40	5	0	0	0	0
Kruskal-Wallis rank test $p < 0.05$.						Kruskal-Wallis rank test $p > 0.05$.					

Comparison of grades of nephropathy among four age groups were made within the same animal group by Kruskal-Wallis rank test. Grades of nephropathy were graded on a scale of 0 through IV. Grade 0, normal glomerulus; Grade I, local lesion within each glomerulus and focal within the kidney; Grade II, diffuse mesangial thickening within the glomerulus and generalized through the kidney; Grade III, capillary lumens are narrowed and only locally obliterated; Grade IV, general luminal narrowing.

(Table 2, Mann-Whitney's U-test). No IgA deposits were found in female diabetic or control mice. The degrees of IgA deposition are summarized in Table 2.

Electron microscopy confirmed the results of light microscopic evaluations of the degrees of glomerular lesions such as glomerular basement membrane thickening, increases in the amount of mesangial matrix, and sclerotic nodular changes in the glomerular tufts (data not shown). Diffuse effacement of the foot process

was observed in all cases (data not shown).

Nephropathy in C3H congenic mice

We investigated the effects of H-2 alleles on development of IgA nephropathy, since MHC has been reported to determine susceptibility to IgA nephropathy in humans (Berthouix et al. 1978; Brettle et al. 1978; Sabatier 1979; Li et al. 1991; Ibels et al. 1994; Fennesey et al. 1996). We compared the natural course of the progression of nephropathy in diabetic males with C3H

Fig. 5. Immunofluorescent staining of IgA deposition in the mesangial area of glomeruli from a male diabetic B6 mouse (A) and male control B6 mouse (B) at 40 weeks of age.

TABLE 2. *Immunofluorescence intensity of glomerular IgA deposition in diabetic and control male mice*

Diabetic						Control								
B6	Grade (No of mice)					B6	Grade (No of mice)							
Age (weeks)	0	I	II	III	Total	Age (weeks)	0	I	II	III	Total	Mann	Whitney's	U-test ¹
10	2	2	1	0	5	10	5	0	0	0	5	$p < 0.05$		
20	0	0	4	1	5	20	3	2	0	0	5	$p < 0.01$		
30	0	0	2	3	5	30	4	0	1	0	5	$p < 0.05$		
40	0	0	2	3	5	40	4	0	1	0	5	$p < 0.05$		
² $p < 0.05$						² $p > 0.05$								
C3H	Grade (No of mice)					C3H	Grade (No of mice)							
Age (weeks)	0	I	II	III	Total	Age (weeks)	0	I	II	III	Total	Mann	Whitney	U-test ¹
10	0	1	4	0	5	10	3	1	1	0	5	$p > 0.05$		
20	0	1	4	0	5	20	2	3	0	0	5	$p < 0.05$		
30	0	0	2	3	5	30	3	2	0	0	5	$p < 0.01$		
40	0	0	1	3	4	40	3	1	1	0	5	$p < 0.05$		

² $p < 0.05$

² $p > 0.05$

¹ Grades of IgA deposition within the same age groups were compared using Mann-Whitney U-test.

² Grades of IgA deposition among different age groups were compared using Kruskal-Wallis rank test.

³ Intensity of glomerular IgA deposition was graded on the scale described in the "Material and Methods" section.

background (5 mice per age per group) and those with B6 background.

The serum BUN levels began to linearly increase from 20 to 40 weeks of age (Fig. 1A) and serum IgA concentrations significantly elevated in diabetic mice after 20 weeks of age (Fig. 3).

Histological analyses revealed similar degrees and types of diabetic lesions in diabetic mice with a C3H background to those with a B6 background (data not shown). The grades of diabetic nephropathy and intensity of IgA deposition did not differ significantly between the two backgrounds within the same age groups (compared by Mann-Whitney's U-test, $p > 0.05$, Tables 1 and 2). These results indicated that MHC did not modify the progression of IgA nephropathy in the present model.

DISCUSSION

Renal complications in diabetics are a serious problem in respect to quality of life and also from the viewpoint of medical expenditures. In Japan, during the onset of clinically overt diabetes, 30 to 40% of patients each year develop diabetic complications and more than 10 000 patients undergo dialysis annually (Maeda 1999). Therefore, the prevention of renal complications is a necessity to prevent enormous increases in medical expenditures.

To date, more than a dozen animal models of diabetes have been reported as developing diabetic nephropathy during the natural course of diabetes (Velasquez et al. 1990). In these mice, histological examinations showed glomerulosclerosis with IgG deposition as the major type of immunoglobulin (Cohen et al. 1987). Some studies, indicated that IgA deposition occurred, albeit to a much lesser extent than IgG deposition (Hashimoto 1986; Miller et al. 1988). In the present study, we confirmed that diabetic nephropathy developed during the natural course of diabetes. The morphological features in this model were similar to those of humans as it included such aspects as nodular,

diffuse, hyalinosis and tubulointerstitial lesions. These lesions progressed gradually and impaired renal functions at the end stage. However, due to technical difficulties, we could not determine any mouse specific albumin in urine. Thus, we could not confirm whether the Akita mouse had albuminuria, a very sensitive marker for predicting clinical stages of diabetic nephropathy in humans.

Diffuse glycogen deposit in proximal tubuli is suggestive of extensive hyperglycemia in the Akita mouse. Such a finding is rarely found in humans except those under poor glycemic control. However, the finding is in accord with a notion that the Akita mouse is an extreme animal diabetic model.

Another unique feature of nephropathy was that IgA was the only class of immunoglobulin, deposited in mesangial areas. From definitions of histological features (Berger 1968), the renal lesions were very similar morphologically to IgA nephropathy (Ibels and Gyory 1994). The elevation of serum IgA in diabetic mice was also consistent with IgA nephropathy. The observation that IgA nephropathy developed to a comparable degree in diabetic males with B6 and C3H backgrounds argued against a crucial role for MHC in IgA nephropathy; although MHC alleles have been suggested to be involved in predisposition to IgA nephropathy in humans (Berthouix et al. 1978; Brettle et al. 1978; Sabatier 1979; Li et al. 1991; Ibels and Gyory 1994; Fennessey et al. 1996). The present results indicated that MHC is not a susceptibility factor for IgA nephropathy associated with diabetes, which is in accord with another report (Gharavi et al. 2000).

In humans, there have been several reports of serum IgA elevation (Ohmuro et al. 1993) and/or IgA nephropathy associated with diabetes. Mak et al. (1997), Orifila et al. (1998) and Kanauchi et al. (2000) reported that 10 to 20% of diabetes cases were complicated with IgA nephropathy as determined by renal biop-

sy; with prevalence rates significant higher than those in the normal population. These observations indicate that, IgA nephropathy is not merely a concurrent complication occurring by chance in diabetic nephropathy but rather is physiologically associated with diabetes even in humans.

In conclusion, the unique features of the present mouse model will be useful to explore the mechanisms of nephropathy as a complication of diabetes as well as investigating the specific forms of nephropathy such as IgA.

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