NOTE

Immunization with ACE (Angiotensin Converting Enzyme) Develops Diabetic Changes in the Kidney and Retina in Diabetogenic Rats

SEIKOH NISHIDA, TAMAKI SASAKI*, HISASHI KIMURA***, JUNJI TANAKA**, TSUTOMU NOHNO#, YASUSHI HIROKAWA, MICHIHIRO MATSUKI AND KIYOSHI ICHIHARA##

Division of Endocrinology, Department of Medicine, Kawasaki Medical School, Okayama 701-0192, Japan
*Division of Nephrology, Department of Medicine, Kawasaki Medical School, Okayama 701-0192, Japan
**Division of Cardiology, Department of Medicine, Kawasaki Medical School, Okayama 701-0192, Japan
***Department of Ophthalmology, Kawasaki Medical School, Okayama 701-0192, Japan
#Department of Molecular Biology, Kawasaki Medical School, Okayama 701-0192, Japan
##Department of Clinical Pathology, Kawasaki Medical School, Okayama 701-0192, Japan

Abstract. In normal New Zealand white rabbits, immunization with rabbit lung ACE (angiotensin converting enzyme) induced atherosclerotic retinal changes, and glomerular changes similar to those seen in diabetic nephropathy. Also, in genetically diabetogenic rats, immunization with the rabbit lung ACE induced diabetic nephropathy and retinopathy.

Key words: Immunization with rabbit lung ACE, Retinal change, Glomerular change, Rabbits and rats

Materials and Methods

The Animal Research Committee of the Kawasaki Medical School approved the following studies on animals.

1. Studies on rabbits

Nine NZ white female rabbits fed RC4-Oriental Yeast containing 0.18 g Na and 1.87 g K per 100 g were immunized with rabbit lung ACE from Sigma after purification by polyacrylamide gel electrophoresis. Monthly injections were administered with 150 μg of the purified ACE dissolved in saline and emulsified with an equal volume of complete Freund’s adjuvant (Gibco) intradermally at multiple sites in the
back and soles. After initial immunization, the rabbits were boosted every 2 weeks by injecting 10 μg of the ACE into the ear vein. In 2 or 3 days following the booster injection, the rabbits were bled once a month and then sera were tested for production of the antibody to ACE by ELISA (enzyme-linked immunosorbent assay). The results were compared with those of 2 rabbits immunized with complete Freund’s adjuvant alone or those of 2 untreated, weight-, and sex-matched rabbits bled randomly for 8 months (control values).

Retinas were examined by ophthalmoscope and fundus camera after tropicamide treatment from the 6th immunization. The examinations were terminated at the 15th immunization when eyeballs and/or the kidneys were extirpated for histological examinations. Color pictures of retinas were taken by a Kowa Genesis camera (K9L22J) and FAG (fluorescein angiography) photos were taken by a set of cameras (Kowa, K9L-FL22J) after a bolus iv injection of 2 ml fluoresce (Alcon, Japan) into the ear vein. Retinal tissues were fixed by 5% formaldehyde solution in 0.1 M PBS (phosphate buffer solution), pH 7.2 and PAS (periodic acid Schiff) stained. The kidneys were fixed by 10% formaldehyde solution and PAS stained for light microscopic examination and were fixed by 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4 (1st fixation) and osmium (VIII) oxide solution (2nd fixation) for electronmicroscopic examination. Immunoperoxidase staining of retina was performed in rabbits and rats at Special Reference Laboratories (Tokyo, Japan) as reported previously [10]. Cryosections were permeabilized with absolute ethanol, and then exposed to microwave irradiation for 10 min in 10 mM citric buffer, pH 6.0, at 98°C, or treated with 0.05% protease (Sigma, type XXVII), at 37°C, for 30 min for activation of the antigen. Next, sections were treated for 5 min with hydrogen peroxidase and blocked with 10% porcine serum. After washing, sections were incubated with goat anti-rabbit (or rat) IgG (Bethyl Lab) or goat anti-rabbit (or rat) IgM (Bethyl Lab), 1 : 100, 4°C overnight. Anti-goat IgG (SBA Inc.) conjugated to horseradish peroxidase (DAKO) was used as secondary antibody. For staining, incubation for 7 min in 3, 3’-diaminobenzidine containing H2O2 was used. Nuclear staining was done with haematoxylin. ELISA for anti-rabbit ACE antibodies was done as reported [4] using the anti-rabbit (or rat) IgM antibody or IgG antibody conjugated with HRP purchased from DAKO A/S, Denmark.

2. Studies on rats

OLETF rats and LETO rats (kindly supplied by Otsuka Pharmaceutical Co., Japan) were fed MF-Oriental Yeast containing 0.26 g Na and 0.89 g K per 100 g and were then immunized with the rabbit lung ACE. OLETF (Otsuka Long-Evans Tokushima Fatty) strain, an animal model of human type 2 diabetes, was established at Otsuka Pharmaceutical Co. from the Long-Evans rat. The characteristics of this strain are the late onset and chronic course of hyperglycemia, hyperlipidemia, mild obesity and renal complications resembling those of human type 2 diabetes [11]. The LETO (Long-Evans Tokushima Otsuka) rat, a control strain of the OLETF rat, and the OLETF rat originated from the same colony of Long-Evans rats, but the LETO line has not shown diabetes mellitus. The rats were divided into rat [I]; LETO rats, rat [II]; LETO rats immunized with ACE, rat [III]; OLETF rats and rat [IV]; OLETF rats immunized with ACE.

Monthly immunizations were done by injecting 100 μg of the purified ACE and booster injections (10 μg) were given every 2 weeks into the tail vein. The results were compared with those of 2 LETO and 2 OLETF rats immunized with adjuvant alone or those of untreated LETO and OLETF rats (controls).

Results

In 3 out of the 9 immunized rabbits, the anti-ACE antibody was detected after 4 months and finally in 8 of the 9 rabbits the antibody became positive by 8 months. Of these 8 rabbits, one rabbit died at 10 months, 3 rabbits were found to have no unequivocal renal changes, while 4 out of the other 5 rabbits demonstrated remarkable changes in the retinal vessels (Fig. 1 and 2). No significant histological changes occurred in the kidney and retina in either control rabbits or control rats after immunization with only Freund’s complete adjuvant. Two immunized rabbits shown in Figs. 1 and 2 demonstrated high titers of antibodies (25 or 26 times higher compared with normal pooled serum) and remarkable glomerular and retinal vessel changes. There were no significant differences between control (n = 2) and immunized (n = 9) rabbits in early morning plasma glucose (103.5 ± 11.5 mg/dl and
Fig. 1. Photos a and b of a control rabbit demonstrate normal glomerulus and tubulointerstitium in PAS staining (a, × 100) and electron microscopic examination (b, × 3000). PAS staining of immunized rabbits (× 100); rabbit 1 (c) demonstrates PAS-positive materials mainly at the mesangium region. Rabbit 2 (e) demonstrates PAS-positive materials mainly at the mesangium region. The expansion of mesangial region and a decrease in mesangial cells together form a nodular lesion similar to that in diabetic nephropathy. Electron microscopic examination (d and f, × 3000); rabbit 2 (f) shows an increase in mesangial matrix and low electron-dense deposits in the mesangial region.

Fig. 2. Normal retina of control rabbit is shown in fundus photography (a), FAG (b) and immunostaining (c, × 50). The immunostaining shows no significant staining of retinal vessel for rabbit IgG. Photos d-f are for rabbit 1 and g-i are for rabbit 2 after the 15th monthly immunization with ACE. Increases in arterial reflexes (d and g), variations in venous caliber, and the dilation, engorgement and tortuosity of the veins (d, e, g and h) are indicative of atherosclerotic changes. Photos f and i demonstrate remarkable staining of retinas for rabbit IgG from ganglionic cell layer to outer nuclear layer.
Fig. 3. A 68 week-old, unimmunized LETO rat weighing 590 g demonstrates normal glomerulus in PAS-staining (a, × 400) and a 68 week-old LETO rat weighing 500 g, treated with ACE demonstrates expansion of PAS-positive mesangial matrix and thickening of lumen wall (b, × 400). A 68 week-old, unimmunized diabetic OLETF rat weighing 530 g demonstrates the increase in mesangial matrix and the deposit of PAS-positive materials in it, which perform segmental sclerosis (c, × 400). A 68 week-old diabetic OLETF rat weighing 575 g, immunized with ACE demonstrates a chronic thickening of lumen wall and expansion of the mesangial matrix where cell infiltration and, partly, nodular lesions are seen (d, × 400).

Fig. 4. A 68-week-old, unimmunized LETO rat weighing 590 g and a 68 week-old LETO rat weighing 500 g, immunized with ACE demonstrate almost normal fundi in fundus photography (a, d), and FAG (b, e). Remarkably positive immunoreactivity for IgM is shown in retinal microvessels of the immunized rat from nerve fiber layer to inner nuclear layer (f, × 50). The immunostaining for rat IgG in the unimmunized LETO rat is weak and equivocal (c, × 50). A 68 week-old, unimmunized diabetic OLETF rat weighing 530 g demonstrates the increase in arterial reflexes (g) and slight dilation of the veins (h) which are indicative of atherosclerotic changes, and the dilation of the capillaries (h) which is indicative of diabetic change. The retinal immunoreactivity for IgM is equivocal in this spontaneously diabetic OLETF rat (i, × 50). A 68 week-old OLETF rat weighing 575 g, immunized with ACE demonstrates the increase in arterial reflexes, slight variations in arterial caliber and the abnormal arteriovenous crossing (j), and the dilation and tortuosity of the veins (k), indicative of atherosclerotic changes. In FAG (k) a few microaneurysms and capillary dilation are seen, suggestive of more apparent diabetic changes of retinal vessels in the immunized OLETF rat than those in the unimmunized OLETF rat. Immunocomplexes for rat IgM are deposited throughout the retinal microvessels from nerve fiber layer to inner nuclear layer (l, × 50).
IMMUNIZATION WITH ACE IN DIABETOGENIC RATS

The mean body weight of the rats was 280 g for LETO rats and 350 g for OLETF rats at 10 weeks old, but after the 14th immunization the same weight of 560 g was shown for both groups. In rats [II] and [IV], circulating antibodies to ACE were detected from the 4th immunization on. In all rats [II] and [IV] examined, titers of the serum antibodies reached a very high plateau (more than $2^8$ times) after the 6th immunization. In rats [III] and [IV] (OLETF rats) histological changes in the kidney were observed from 10 months on. Also in rat [II] (LETO rat) renal histological changes were demonstrated after immunization (Fig. 3). Photographic changes of the retinal vessels were seen in rats [III] and [IV] from 10 months and the changes were most remarkable in rats [IV] (Fig. 4).

As shown in Fig. 5, retinal capillaries in immunized rats showed a variety of atherosclerotic changes, including thickening of the wall, dilation, infiltration of plasma cell or lymphocyte in adjacency, or protrusion into vitreous cavity or protrusion of wall into the capillary cavity. In rats [III] and [IV] diabetic states were superinduced to the atherosclerotic vessel changes. In most of the OLETF rats, cataracts occurred from 15 months, and retinal vessel changes could not be evaluated. There were no significant differences between rats [I] ($n = 5$) and rats [II] ($n = 4$) in early morning plasma glucose (172.8 ± 15.6 and 159.5 ± 4.5 mg/dl) and HbA1C (3.0 ± 0.3% and 2.8 ± 0.2%) levels, respectively.

Discussion

On the whole, circulating ACE antibodies were detected after the 4th monthly immunization, and morphological changes in the kidney and retina were demonstrated after the 10th immunization in both rabbits and rats. The glomerular changes of the rabbit induced by the immunization with rabbit lung ACE may have resulted from the renal deposition of the anti-ACE immunocomplex. It is thought that the immunocomplex may induce nephropathy in rabbits, leading to the thickening of the basement membrane, an increase in mesangial matrix and exudative deposits similar to those seen in diabetic nephropathy (Fig. 1). There is evidence in studies of congenitally diabetic mice that mesangial and basement-membrane thickening are accompanied by mesangial staining for IgG in the mesangium and in the glomerular hyaline nodules [12]. In these studies immunofluorescent staining also involved the extraglomerular portion of the mesangium and distal tubular cells. In spontaneously diabetic OLETF rats, positive glomerular immunofluorescence for immunoglobulins was observed before the onset of diabetic nephropathy [13]. In humans as well, the positive basement-membrane immunofluorescence for IgG and albumin in severe diabetic nephropathy clearly distinguishes this group from other renal diseases [14].

The light microscopic changes and the immunopathologic changes thus appear to coexist from an early stage of diabetic nephropathy. This suggests that the renal deposition of immunoglobulins may play a permissive role in the development of diabetic nephro-

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Fig. 5. Capillaries in rat retinas (× 250). a, from unimmunized LETO rats as a control. b–f, from immunized LETO and OLETF rats. A variety of atherosclerotic changes are demonstrated in b–f, including thickening of wall (deposited with PAS-positive materials), dilation, infiltration of plasma cell or lymphocyte in adjacency, protrusion into vitreous cavity, and protrusion of wall into capillary cavity forming aneurysm.
pathy, since diabetic glomerular disease is a manifestation of a fundamental metabolic disturbance [12]. The differences in the morphology of diabetic glomerular disease in various animals and humans may represent species differences in response to this metabolic disturbance as well as differences in the duration of disease.

It is apparent that the immunization of control (LETO) and diabetic (OLETF) rats with ACE induced the development and progression of renal changes (Fig. 3). In control rats, the immunization induced the expansion of PAS-positive mesangial region and the thickening of the lumen wall, and in diabetic rats, it induced diffuse thickening of the lumen wall and expansion of the mesangial region where it was accompanied by the cell infiltration and formation of nodular lesions. These findings suggest that when the renal changes in immunized LETO rats (immunization with ACE) are superinduced to those in unimmunized OLETF rats (diabetic state), the process of diabetic nephropathy is brought to completion as seen in the typical diabetic nephropathy of spontaneously diabetic OLETF rats. The microscopic changes in the kidney may coexist with the renal deposition of immunoglobulins in rats.

In a previous report it was demonstrated that the glomerular localization of immunoglobulins and complement occurs in OLETF rats [13]. It is clear that immunopathologic changes are a necessary cofactor for diabetic glomerulopathy in rats, and that glomerulopathy is secondary to the abnormal metabolic environment in which the kidney resides [12]. However, it needs to be elucidated whether diabetic nephropathy occurs only in the hyperglycemic state or whether it can occur without the renal deposition of immunoglobulins, as there are many reports dealing with the localization of renal immunoglobulins and/or complement (C3) in diabetic rats that are not necessarily specifically immunologic for injury [14].

The occurrence of diabetic retinopathy has been shown in spontaneously diabetic OLETF rats. In a review Kawano et al. [11] pointed out retinal complications in OLETF rats, though no data was shown. In one of the 4 OLETF rats, at 80 weeks old, a hard exudate was demonstrated in the retina [15]. Examination by electroretinogram indicated the development of functional diabetic retinopathy in 40 week-old OLETF rats, though there were no diabetic changes in ocular fundi [16]. Microscopic examination in 19 week-old OLETF rats revealed thickening of the basement membrane of the retinal capillary wall, loss and degeneration of pericytes, and a dilated and twisted venous wall [17]. From about 50 week-old, retinal vessel changes as well as renal changes were demonstrated in diabeticogenic OLETF rats in the present study. Cataracts were also seen at the same age. Diabeticogenic OLETF rats that were immunized with ACE showed remarkable vessel changes of the retina in FAG or fundus photography in comparison with the retinal changes in unimmunized OLETF rats or immunized control LETO rats. From the literature concerning the pathogenesis of diabetic retinopathy, it is suggested that endothelial damage may be a trigger for the development of the disease. Endothelial damage and hyperglycemia interact to cause increased blood flow and capillary damage [18]. Dysfunction of the endothelium forms the cellular basis for microvascular leakage of plasma protein in human diabetic retinopathy [19]. Vascular endothelial growth factor (VEGF) is a specific growth factor for endothelial cells and VEGF mRNA is expressed in the vessel wall of retinas of diabetic humans and rats [20, 21]. Thus, hyperglycemia, endothelial damage and resultant vessel changes may interact to develop diabetic retinopathy. It is of interest to note that, with further immunizations, immunodeposition may induce atheroslerotic changes of the retinal vessels (Fig. 5). This retinal immunodeposition is probably the ACE immunocomplex, because it is not produced in unimmunized rats. The superinduction of the ACE immunocomplex to hyperglycemia may thus result in diabetic retinopathy via the same mechanism by which diabetic nephropathy develops. Hansson et al. [9] have stated in a review that autoantibodies to lipoproteins, lipids or other components of the arterial wall can be formed during the course of an atherosclerotic disease, and that molecules released after endothelial damage could be presented to T lymphocytes by local antigen presenting cells. In fact, several types of antibodies have been shown to elicit antibody responses related to atherosclerosis.

We postulated that the ACE antibody may increase susceptibility to atherosclerosis, and that if diabeticogenic characteristics are superinduced to atherosclerotic changes, diabetic vascular complications may appear. In fact, plasma total cholesterol and triglyceride levels are remarkably elevated in OLETF rats in comparison with LETO rats [11].
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