Supplementary material to Bruno et al. “Immune complex-mediated glomerulonephritis is ameliorated by thrombin-activatable fibrinolysis inhibitor deficiency” TH-08-02-0092

Materials and Methods

TAFI knockout mice, originally generated and characterized by Nagashima et al, were backcrossed with C57BL/6 mice for more than ten generations before use in these experiments [1]. Mice were housed on constant 12-hour light/12 hours dark cycle in a temperature and humidity-controlled room, and were given free access to standard chow and water. Female knockout and wild type littermates between 8 and 12 weeks old were used in the experiments. The Mie University’s Committee on animal investigation approved the experimental protocol, and the experiments were performed according to the guidelines for animal experiments of the National Institute of Health.

Animal model of glomerulonephritis

Immune complex-mediated glomerulonephritis was induced as described by Welch et al [2]. Briefly, animals were treated by alternate intraperitoneal injections of 6 mg of horse spleen apoferritin (HSA) (Sigma, St Louis, MO) plus 0.1 mg of lipopolysaccharide (Sigma, St Louis, MO) 3 days per week and 6 mg of horse spleen apoferritin alone 2 days per week over 6 weeks. Control animals were treated with vehicle (physiological saline solution) by the same route for the same time.

Study design

Four groups of animals were treated: wild-type mice (WT/SAL, n=7) or knockout (KO/SAL, n=7) mice treated with intraperitoneal sterile saline (SAL) and wild-type mice (WT/HSA, n=7) or knockout (KO/HSA, n=7) mice treated with intraperitoneal injection of horse spleen apoferritin plus LPS (HSA).

Evaluation of animal model

Mice were individually placed in metabolic cages with free access to tap water and
standard chow on weeks 3, 5 and 6, and urine was collected over 24 hours. Blood was sampled by cardiac puncture on week 7 and collected in tubes containing 1/10 volume of 3.8% sodium citrate. Both urine and blood were then centrifuged (1500 x g at 4°C for 20 min), supernatants aliquoted and stored at -80°C until use. The concentration of albumin in urine was measured using a commercial kit from Wako Pure Chemicals Industries (Chuo-ku, Osaka, Japan), the results were confirmed by measuring the protein to creatinine ratio in samples of fresh voided urine using dip-strip from Bayer® (Bridgend, UK). Total protein in urine and homogenized renal tissue was measured using the BCA protein assay kit from Pierce (Rockford, IL). The blood concentration of urea nitrogen was measured by the urease indophenol method using a commercial kit from Wako Pure Chemicals Industries (Chuo-ku, Osaka, Japan). The plasma concentration of anti-HSA antibodies was measured by enzyme immunoassays as previously described [3]. Briefly, 96-well microtiter plates (Nunc, Apogen, Denmark) were coated at 4°C overnight with 0.1 mg/ml of HSA in phosphate-buffered saline (PBS; 0.15M NaCl, 0.001M NaH3PO4). After appropriate washing with 0.05% Tween-20 (Nakarai Chemical, Japan) in PBS, non-specific protein binding was blocked with 1% bovine serum albumin at room temperature. After washing, 100 µl of 1000-fold diluted plasma was added to the wells in duplicate and incubated for 1h. Then horseradish-peroxidase anti-mouse IgG antibody was added to the wells and incubated for 30 min, before development with peroxidase substrate solution and measuring absorbance at 450 nm using a plate reader (Bio-RAD 550 Hercules, CA.). Pooled plasma from three mice treated with 6 mg of HSA for ten weeks was used for construction of a standard curve.

Necropsy of animals

Mice were sacrificed on week 7 under profound anesthesia by intraperitoneal injection of pentobarbital to take samples for biochemical and histological examinations. The left kidney was excised and frozen at -80°C and the right kidney after excision was fixed in 10% formalin and then embedded in paraffin. Renal tissue samples were then
deparaffinized and 3-µm-thick slides were prepared for hematoxylin-eosin, periodic acid-Schiff, Masson’s Trichrome (Sigma-Aldrich, St Louis, MO) and immunohistochemical staining.

Immunohistochemistry

Immunohistochemical staining of collagen type I and fibrin was performed using rabbit anti-mouse collagen I (Bethyl Laboratories, Montgomery, TX) antibody and rabbit anti-fibrinogen (Dako, Glostrup, Denmark) antibody as described [4]. The samples were then treated with biotin-labeled anti-rabbit IgG, peroxidase-labeled streptavidin, and peroxidase substrate using the catalyzed Signal Amplification System from Dako (Kyoto, Japan). Smooth muscle cells and complement C3 deposits were stained by similar methods using monoclonal anti-caldesmon and anti-mouse C3 (Hycult Biotechnology) antibodies. All staining was performed after appropriate blocking of endogenous renal peroxidase and biotin. The presence of renal deposits of immune complex was investigated indirectly by staining HSA using rabbit anti-HSA (Sigma-Aldrich, St Louis, MO) as primary antibody and fluorescein-isothiocyanate-labeled anti-rabbit IgG (KPL Corporation, UK) as secondary antibody.

Gelatin Zymography

Gelatin zymography was carried out using a commercial kit from Invitrogen Life Technology (Carlsbad, CA). Briefly, renal tissues were homogenized in protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) using a Polytron (Kinematica GmbH, Switzerland) for 2 min. The homogenates were then centrifuged for 15 min (15,000 x g) at 4°C and the supernatants were separated on 10% denaturing gels. Human MMP-2 and MMP-9 (Chemicon International, Temecula, CA) were used as positive controls. The gel was then incubated in developing solution and stained using the Novex Colloidal Blue Staining kit from Invitrogen. Photographs of the gels were analyzed using WinRoof image analysis software.
**Measurement of hydroxyproline**

To determine the total amount of collagen, hydroxyproline content in the renal tissues was measured by a colorimetric method. Briefly, the left kidney was excised, dried and then homogenized as described above. The samples were then hydrolyzed in 1ml of 6 N HCl for 20 hours at 110 C. Five µl of standards or samples and 5 µl of citrate-acetate buffer (citric-acid 25 g, sodium acetate 60 g, sodium hydroxide 17 g, glacial acetic-acid 6 ml) were applied to a 96-well plate. This then was treated with chloramine T solution (282 mg chloramine T dissolved in 20 ml of a buffer containing 10% n-propanolol, 10% H₂O and 80% citrate-acetate buffer) and incubated for 20 min at room temperature. Ehrlich’s solution (2.5g 4-dimethylaminobenzaldehyde dissolved in a buffer containing 9.3ml n-propanolol and 3.9ml 70% perchloric acid) was added and incubated at 65 C for 20 min. Absorbance was then measured at 550 nm with a plate reader.

**Biochemical analysis**

The concentration of total protein was measured with the BCA™ protein assay kit (Pierce, Rockford, IL) following the manufacturer’s instructions. The concentrations of transforming growth factor-β (TGF-β; BD Bioscience Pharmingen) and osteopontin (R&D Systems, Minneapolis, MN) were measured using commercial enzyme immunoassay (EIA) kits following the manufacturer’s instructions. The level of mouse plasminogen activator inhibitor-1 (PAI-1) was measured by EIA using a primary monoclonal antibody specific for mouse PAI-1 and biotin-labeled anti-PAI-1 antibody. Fibrin(ogen) was determined using rabbit anti-mouse fibrin(ogen) (Dako, Glostrup, Denmark) antibody and biotin-labeled antifibrin(ogen) antibody. C3a-desArg measurement in plasma was performed with a commercial immunoassay kit (Cedarlane, Ontario, Canada). The activity of plasmin was determined using the synthetic substrate S-2251 (Chromogenix, Mölndal, Sweden). In brief, 100 µl of renal homogenate from each group of mice was added to wells of a 96-well plate and then treated with 100 µl (1/10) of the stock chromogenic substrate (4mmol/l). The amount of p-nitro aniline
released was measured at 405 nm after 10, 30 and 60 min of incubation. Standard curves were generated using human plasmin (Haematologic Thechnologies Inc, Essex Junction, VT USA) and then the values were corrected by the protein concentration.

**Figure legends**

**Supplementary Figure 1. Structural changes in juxtamedullary glomeruli.** Tissue preparations (A) were stained with periodic acid-Schiff or with rabbit anti-horse spleen apoferritin followed by FITC-labeled anti-rabbit IgG (B). There is increased PAS (+) deposition and enhanced cellularity in the glomerular mesangial space of WT/HSA and KO/HSA mice compared with WT/SAL and KO/SAL mice. Similar changes were observed in most glomeruli. Immunofluorescence staining shows increased deposition of apoferritin in glomeruli from WT/HSA mice compared to KO/HSA and saline-treated mice. Arrows indicate antigen deposits. Bars on the figures represent 10 m. “G” denotes the glomerulus.

**Supplementary Figure 2. Staining of renal collagen deposits.** Tissue preparations (A) were stained with Masson’s trichrome stain and collagen positive areas (blue) were quantified (B) using WinRoof image analyzer software. n=5–7. Data are expressed as the mean ± s.e.m. Statistical difference between groups was analyzed by ANOVA and *post hoc* using Tukey’s test. Bars on the figures represent 50 m.

**Supplementary Figure 3. Immunostaining of caldesmon.** Tissue preparations (A) were stained with anti-caldesmon antibody and immunoreactive areas (brown) were quantified (B) using the WinRoof image analyzer software (n=4–6). Data are expressed as the mean ± s.e.m. Statistical difference between groups was analyzed by ANOVA and *post hoc* using Tukey’s test. Bars on the figures represent 50 m.

**References**


Supplementary Figure 1
Supplementary Figure 2

A

WT/SAL

KO/SAL

WT/HSA

KO/HSA

B

Masson’s Trichrome Stain (% total area)

WT/SAL  WT/HSA  KO/SAL  KO/HSA

P < 0.001  P < 0.001  NS
Supplementary Figure 3

A

WT/SAL  WT/HSA

KO/SAL  KO/HSA

B

Caldesmon staining (% total area)

WT/SAL  WT/HSA  KO/SAL  KO/HSA

P < 0.001  P < 0.001  P < 0.001