Original Article

Phenotypic change of macrophages in the progression of diabetic nephropathy; sialoadhesin-positive activated macrophages are increased in diabetic kidney

Ryo Nagase[1#], Nobuo Kajitani[1#], Kenichi Shikata[1,3], Daisuke Ogawa[1,2], Ryo Kodera[1,3], Shinichi Okada[1], Yuichi Kido[1], and Hirofumi Makino[1]

Departments of [1]Medicine and Clinical Science, and [2]Diabetic Nephropathy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, [3]Center for Innovative Clinical Medicine, Okayama University Hospital, Okayama, Japan.

Running Title: Phenotypic change of macrophages in diabetic nephropathy

Keywords: macrophage, sialoadhesin, diabetic nephropathy

Correspondence to: Kenichi Shikata, M.D., Ph.D. and Daisuke Ogawa, M.D., Ph.D.
Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences
2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan
Phone: (+81) 86-235-7235
Fax: (+81) 86-222-5214
E-mail: shikata@md.okayama-u.ac.jp, daiogawa@md.okayama-u.ac.jp

[#] The first and second authors equally contributed to this work.

Abstract

Background

Inflammatory process is involved in pathogenesis of diabetic nephropathy, although the activation and phenotypic change of macrophages in diabetic kidney has remained unclear. Sialoadhesin is a macrophage adhesion molecule containing 17 extracellular immunoglobulin-like domeins, and is an I-type lectin which binds to sialic acid ligands expressed on hematopoietic cells. The aim of this study is to clarify the activation and phenotypic change of macrophages in the progression of diabetic nephropathy.

Methods

We examined the expression of surface markers for pan-macrophages, resident macrophages, sialoadhesin, MHC class II and α-smooth muscle actin in the glomeruli of diabetic rats using immunohistochemistry at 0, 1, 4, 12, and 24 weeks after induction of diabetes by streptozotocin. Expression of type IV collagen and the change of mesangial matrix area were also measured. The mechanism for up-regulated expression of sialoadhesin on macrophages was evaluated *in vitro*.

Results

The number of macrophages was increased in diabetic glomeruli at 1 month after induction of diabetes and the increased number was maintained until 6 months. On the other hand, sialoadhesin-positive macrophages were increased during the late stage of diabetes concomitantly with the increase of α-smooth muscle actin positive mesangial cells, mesangial matrix area and type IV collagen. Gene expression of sialoadhesin was induced by the stimulation with IL-1β and TNF-α but not with IL-4, TGF-β and high glucose in cultured human macrophages.

Conclusion

The present findings suggest that sialoadhesin-positive macrophages may contribute to the progression of diabetic nephropathy.

Introduction

Diabetic nephropathy is a leading cause of end-stage renal failure in developed countries. Infiltration of mononuclear cells is a characteristic of the glomeruli in the patients with diabetes (1). We previously demonstrated that intercellular adhesion molecule-1 (ICAM-1) mediates macrophage infiltration into the glomeruli of streptozotocin (STZ) -induced diabetic rats (2). Furthermore, we demonstrated that infiltration of macrophages was suppressed in diabetic ICAM-1 knockout mice and urinary albumin excretion, glomerular hypertrophy and mesangial matrix expansion were significantly suppressed in diabetic ICAM-1 knockout mice than in diabetic wild type mice (3). These findings strongly suggest the important role of macrophages in the progression of diabetic nephropathy, although the state of activation and the phenotype of macrophages infiltrated into diabetic glomeruli have remained unclear.

Sialoadhesin (Sn) is a sialic acid-dependent lectin-like receptor (4) which mediates cell to cell interactions. Expression of Sn is normally restricted to the distinct subsets of tissue macrophages including lymphoid tissue macrophage (5). Sn is induced rapidly in response to serum factors (6), glucocorticoids, and cytokines (7). Recent studies in humans have shown that Sn is expressed abundantly on macrophages in the pathological tissues of multiple sclerosis, atherosclerosis, rheumatoid arthritis, and breast cancer (8), suggesting that Sn-positive macrophages is related to chronic inflammation.

Ito et al. demonstrated that Sn-positive macrophages were observed in the prolonged model of mesangial proliferative glomerulonephritis (9). This study suggests that Sn-positive macrophages might be involved in the chronic inflammation of the kidney. There have been some reports which describe the critical role of

Sn-positive macrophages in the pathogenesis of experimental glomerulonephritis (10-12), however little is known about Sn-positive macrophages in the pathogenesis of diabetic nephropathy. To elucidate the relationship between Sn-positive macrophages and diabetic nephropathy, we analyzed the phenotypes of macrophages in diabetic glomeruli by immunohistochemically examining ED1 (pan-macrophages marker), ED2 (resident macrophages marker), ED3 (Sn-positive macrophages marker) (13). We also analyzed the activated macrophages which express OX-6 (MHC class II positive cells marker) on the cell surface.

Diabetic nephropathy is characterized histologically by glomerular hypertrophy, glomerular basement membrane thickening, mesangial matrix expansion, and ultimately glomerular sclerosis (14-17). In diabetic glomerulosclerosis, there is an accumulation of the matrix proteins, type IV collagen or fibronectin (18-19). Because the mesangial cells are responsible for this matrix protein synthesis, overproduction of these matrix proteins is considered to be a result of phenotypic change in the masangial cells (18). Therefore, we also analyzed α-smooth muscle actin (α-SMA) as a marker of the phenotypic change of the masangial cells (20-21). Furthermore, we examined the expression of ICAM-1, type IV collagen, and mesangial matrix area.

Materials and Methods

Animals

Male Sprague Dawley (SD) rats were purchased from Charles River Japan (Yokohama, Japan). Male SD rats weighting 120 g (4 weeks of age) were used in this study. These rats received a standard chow and water diet. All procedures were performed according to the Guidelines for Animal Experiments at Okayama University Medical School, Japanese Government Animal Protection and Management Law (No. 105) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6).

Induction of diabetes

Diabetes was induced in 25 rats by the intravenous injection of 65 mg/kg STZ (Sigma-Aldrich, St. Louis, MO) in 10 mM citrate buffer solution (pH 4.5). The control rats were injected with citrate buffer alone. Blood was collected from a tail vein and assayed for glucose. Urinary protein was determined by the biuret method. Five diabetic and five control rats were sacrificed under anaesthesia and the kidneys were harvested at 1, 4, 12, 24 weeks after the injection with STZ or buffer. Kidneys were weighed and fixed in 10% formalin for periodic acid-methenamine-silver (PAM) staining and the remaining tissues were embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan) and immediately frozen in acetone cooled on dry ice. Metabolic data were measured as described previously (2).

Metabolic data

Blood glucose, urinary albumin excretion (24 hours), and body weight were measured at 0, 1, 4, 12, and 24 weeks. Urine collection was performed for 24 hours with each rat individually housed in a metabolic cage and having free access to food and water. Blood glucose was measured by the glucose oxidase method. Urinary albumin concentration was measured by nephelometry (Organon Teknika-Cappel, Durham, NC). Glycosylated hemoglobin (HbA1c) was measured at 0, 1, 4, 12, and 24 weeks after induction of diabetes by latex-agglutination assay.

Effect of Insulin treatment

Three days after the administration of STZ, when all animals (n=5) exhibited blood glucose levels >300mg/dl, insulin treatment was initiated using nearly 24 hour acting Humalin N (Shionogi, Osaka, Japan) as described previously (2). All insulin-treated rats were sacrificed at 4 weeks and processed for immunohistochemical study.

Antibodies

As primary antibodies, we used mouse antibodies against rat pan-macrophages (ED1), resident macrophages (ED2), sialoadhesin-positive macrophages (ED3), and MHC class II positive cells (OX6), and these antibodies were purchased from AbD Serotec (Oxford, UK). Mouse anti-rat ICAM-1 monoclonal antibody was purchased from Seikagaku Corporation (Tokyo, Japan). Rabbit anti-mouse collagen IV antibody was purchased from LSL (Tokyo, Japan). Mouse anti α-SMA monoclonal antibody was purchased from Oncogene (Boston, MA).

As secondary antibodies, biotinylated goat anti mouse IgG and fluorescein isothiocyanate (FITC)-labeled goat anti mouse IgG were obtained from Jackson

Immunoresearch Laboratories (West Grove, PA). Rodamin-labeled anti mouse IgG was obtained from Chemicon International (Temecula, CA). FITC-labeled anti rabbit IgG was obtained from Zymed Laboratories (San Francisco, CA).

Histopathological examination

Immunoperoxidase and immunofluorescence staining were performed as described previously (2). Fresh frozen sections were cut at 4-μm thickness using a cryostat. To evaluate the phenotype of infiltrated macrophages, anti-ED1, ED2, and ED3 antibodies were applied to the fresh frozen sections as the primary reaction, followed by a second reaction with rodamin-labeled anti-mouse IgG antibody. Then FITC-labeled anti-rat OX-6 antibody was double stained. Intraglomerular ED1, ED2, ED3, and OX-6 positive cells were counted in 10 glomeruli per animal (total 50 glomeruli for each group). The average number per glomerulus was used for the estimation. ICAM-1 and type IV collagen were also detected by the indirect immunofluorescence method. Briefly, sections were fixed with cold acetone for 3 min and stained with each monoclonal antibodies for 24 h at 4°C. Then, the sections were stained with each FITC-labeled anti IgG antibody for 30 min at room temperature. The sections were washed in PBS, mounted with PermaFluor (Shandon, Pittsburgh, PA) and examined under a fluorescence microscope (LSM-510, Carl Zeiss, Jena, Germany). The intensity of ICAM-1 and type IV collagen in the glomeruli was evaluated semi-quantitatively from 0 to 3+.

The distribution of α-SMA was evaluated by immunoperoxidase assays using Vectastain (Vector, Burlingame, CA). In brief, the frozen sections (4-μm thick) were fixed with cold acetone for 3 min and nonspecific protein binding was blocked by

incubation with normal goat serum and avidin for 20 min. The sections were first incubated with each monoclonal antibodies for 24 hours at 4°C. Then the sections were incubated with biotin-labeled anti IgG antibody for 30 min at room temperature. Endogenous peroxidase activity was blocked by incubating the sections in methanol containing 0.3% H_2O_2 for 30 min. After that, the sections were stained with a Vectastain ABC kit. The sections were then counterstained with Mayer's hematoxylin and the percentage of α-SMA-positive glomeruli was evaluated.

Light microscopy

Renal tissues were fixed in 10% formalin and embedded in paraffin in a routine fashion. Tissue sections were cut at 4-μm thickness, dewaxed and stained with PAM. To evaluate glomerular size, randomly selected 5 glomeruli in the cortex per animal were examined under high magnification (x200). Mesangial matrix area was defined as the PAM-positive area within the tuft area and was measured using Photoshop software Ver. 6 (Adobe Systems, San Jose, CA) and analyzed by Scion Image Ver.4.0.2. The results are expressed as mean ± SEM (μm^2).

Cell culture

The human monocytic cell line THP-1 (Japanese Collection of Research Bioresources, Tokyo, Japan) was cultured in RPMI-1640 (GIBCO/Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% (vol/vol) fetal bovine serum (Thermo Fisher Scientific, Waltham, MA). Cells were cultured at 37°C in humidified air containing 5% CO2. For experiments, the cells were adjusted to the cell density of 10^6 cells/ml in the same culture medium. Cells were stimulated with recombinant human tumor necrosis

factor-α (TNF-α: 0.01 μg/l to 10 μg/l) (R&D systems, Mineapolis, MN), recombinant human interleukin-1β (IL-1β: 0.01 μg/l to 10 μg/l) (R&D systems), recombinant human IL-4 (10 μg/l) (R&D systems) and recombinant human transforming growth factor-β1 (TGF-β1: 10 μg/l) (R&D systems). Cells were also stimulated on hyperglycemic condition (5.4 g/l D-glucose) (Sigma-Aldrich); as an osmotic control, D-Mannitol (3.63 g/l) (Sigma-Aldrich) was added to culture medium in simultaneous wells. Total RNA was extracted from THP-1 cells 24 hours after stimulation.

RNA extraction and Quantitative real-time RT-PCR

Total RNA was extracted using the RNeasy plus mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Single-strand complementary DNA was synthesized from the extracted RNA using a GeneAmp RNA PCR Core kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. To evaluate mRNA expression of Sn in THP-1 cells, and IL-1β and TNF-α in rat kidney, quantitative real-time RT-PCR was performed using a Light Cycler (Roche Diagnostics, Tokyo, Japan) and SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) as previously described (22). The mRNA expression was normalized with a house-keeping gene (GAPDH or β-actin) in each sample by calculating the relative expression ratio. For amplification of the complementary DNA, the following oligonucleotide primers were purchased from Takara Bio: Sn, sense 5′-CTGCGAATCAGGGACCAACA-3′, antisense 5′-TTTCAACCCAAATCCTAGAGCAGAG-3′; IL-1β, sense 5′-GCTGTGGCAGCTACCTATGTCTTG-3′, antisense 5′-AGGTCGTCATCATCCCACGAG-3′; TNF-α, sense 5′-TCAGTTCCATGGCCCAGAC-3′, antisense

5′-GTTGTCTTTGAGATCCATGCCATT-3′; GAPDH, sense 5′-GCACCGTCAAGGCTGAGAAC-3′, antisense 5′-TGGTGAAGACGCCAGTGGA-3′; β-actin, sense 5′-GGAGATTACTGCCCTGGCTCCTA-3′, antisense 5′-GACTCATCGTACTCCTGCTTGCTG-3′. Each experiment was performed twice.

Statistical analysis

All values are expressed as the mean ± SEM. Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Scheffe's test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

Results

Metabolic data

Body weight, kidney weight, urinary albumin excretion, and glycosylated hemoglobin are shown in Table 1. Diabetic rats had a significantly lower body weight and higher kidney weight per body weight from 4 weeks after the induction of diabetes. An increase in urinary albumin excretion was observed after 4 weeks. All diabetic rats were moderately hyperglycemic. The serum HbA1c concentration in the diabetic group was significantly higher than in the control group.

Phenotype of macrophage in the glomeruli

Throughout the experiment, ED1, ED2, ED3 and OX-6 positive macrophages are analyzed in the glomeruli of control rats and of animals with STZ-induced diabetes (Fig. 1). When the sections were incubated with irrelevant mouse IgG, no staining was observed. The glomeruli of control rats showed only a few ED1, ED2, and ED3 positive macrophages. In the rats with STZ-induced diabetes, ED1, which is expressed in pan macrophage, peaked at 1 week and maintained to 24 weeks, and most of this macrophages are positive for MHC class II (OX-6) in the glomeruli. ED2, which is expressed in resident macrophage, was not increased in diabetic glomeruli. Sn-positive macrophage (ED3-positive) continued to increase gradually and most of these macrophages are also positive for MHC class II in the glomeruli (Fig. 1).

Expression of ICAM-1

In normal rat glomeruli, ICAM-1 staining was weakly detected. In the glomeruli of STZ-induced diabetes, ICAM-1 fluorescence intensity increased early after diabetes

induction, reaching a peak at 12 weeks and was significantly higher than in the control animals (Fig. 2). A linear ICAM-1 staining pattern was detectable along the capillary walls and the mesangial area.

Staining for α-SMA

In control rats, α-SMA was expressed in the arterioles but not in the glomerular cells. The rate of α-SMA-positive glomeruli increased gradually and increased particularly from 12 to 24 weeks in diabetic rats (Fig. 3).

Staining for type IV collagen

In control rats, there was weak staining for type IV collagen throughout the experimental period. The expression of type IV collagen increased between 12 weeks and 24 weeks in diabetic rats (Fig. 4).

Mesangial matrix area

Representative glomeruli in PAM stained sections are shown in Fig. 5. Glomerular hypertrophy and mesangial matrix expansion were observed in diabetic rat. In STZ-induced diabetic rats, mesangial matrix area was increased significantly larger than those in control rats (Fig. 5).

Effect of insulin treatment

A significantly higher body weight and lower kidney weight per body weight were observed in the insulin-treated diabetic group than in the untreated diabetic group (Table. 1). After 4 weeks of insulin treatment, macrophages infiltrated into diabetic

glomeruli decreased to the same level of control rats (Fig. 1), and ICAM-1 expression in the diabetic rats was comparable to that in the control rats (Fig.2). The expression of α-SMA and type IV collagen, and mesangial matrix area were also suppressed as same as the control rats (Fig. 3-5). These results indicated that hyperglycemia but not STZ induced the infiltration of macrophages and subsequent histological changes in the glomeruli of diabetic rats.

Effects of cytokines and high glucose on the expression of Sn on THP-1 cells

Sn mRNA expression was significantly increased with concentration dependency by the stimulation of IL-1β and TNF-α (Fig. 6). However, Sn expression was not induced by hyperglycemic condition or stimulation with IL-4, and TGF-β1. To confirm whether IL-1β and TNF-α is upregulated *in vivo*, we analyzed the gene expression of IL-1β and TNF-α in the glomeruli of control and diabetic rats. As shown in Fig. 7, mRNA expression of IL-1β and TNF-α is upregulated in diabetic glomeruli. These results suggest that Sn might be induced by these inflammatory cytokines *in vivo*.

Discussion

Sn is expressed on a subpopulation of macrophages with a highly restricted tissue distribution in normal conditions including marginal zone macrophages of the spleen, sinus macrophages of lymph nodes, and omentum macrophages (23). It is important to note that Sn can be rapidly induced in response to serum factors (6), glucocorticoids, and cytokines (7). Recent studies in humans have shown that Sn can be expressed abundantly on macrophages recruited during pathological conditions including multiple sclerosis, atherosclerosis, rheumatoid arthritis, and breast cancer (8). Therefore, Sn is suggested to relate to the chronic inflammation and considered as a marker of activated macrophages.

Only a few macrophages are seen in the glomeruli of non diabetic control rats. On the other hand, ED1 which is expressed in pan macrophages, peaked at 1 week and maintained to 24 weeks, and most of macrophages are positive for MHC class II in the glomeruli (Fig. 1C and 1G). ED2 which is expressed in resident macrophages in the rats with STZ-induced diabetes (13), did not increase in diabetic glomeruli (Fig. 1H). Sn-positive macrophages continued to increase gradually and most of these macrophages are also positive for MHC class II in the diabetic glomeruli (Fig. 1F and 1I). Since ICAM-1 expression was significantly upregulated in the diabetic rats compared to the control animals (Fig. 2), ICAM-1 may be involved in the infiltration of total and Sn-positive macrophages in diabetic glomeruli. Sn-negative activated macrophages are infiltrated in the glomeruli in the early phase of diabetic nephropathy, but the proportion of Sn-positive activated macrophages was increased during the progression of diabetic nephropathy. These results indicated that Sn-positive macrophages may contribute to the chronic inflammation in diabetic nephropathy.

We further investigated the correlation of phenotypic change between macrophages and mesangial cells in the glomeruli of STZ-induced diabetic rats. The α-SMA is considered as a marker for the phenotypic change of mesangial cells (20-21) and to play a pivotal role in the accumulation of extracellular matrix in diabetic nephropathy. The rate of α-SMA-positive glomeruli increased gradually and increased particularly from 12 to 24 weeks in diabetic rats (Fig. 3). Further, the expression of type IV collagen increased between 12 weeks and 24 weeks in diabetic rats (Fig. 4), and mesangial matrix area in diabetic rats was increased significantly larger than those in control rats (Fig. 5). Therefore, the phenotypic change of mesangial cells and accumulation of extracellular matrix was observed in parallel with increase of Sn-positive macrophages.

Sn was originally defined as a sheep erythrocyte receptor on mouse macrophages (5-6). In both mouse and human, the cDNA for Sn encodes a 185-kDa type I transmembrane glycoprotein, made up of 17 immunoglobulin-like domains and a short cytoplasmic tail (24-25). Sn is a prototypic member of the Siglec family of sialic acid-binding immunoglobulin-like lectins and is also referred to as Siglec-1 (26). Sn was designed CD169 at the seventh human leukocyte differentiation antigen workshop. The large extracellular region of Sn contains a sialic acid-binding site within the membrane-distal V-set domain and is assumed to extend away from the glycocalyx on the cell surface to mediate cell to cell interactions (27). To investigate the mechanism of the induction of Sn in macrophages, gene expression of Sn was induced by several conditions in THP-1 human monocytic cell line. Sn mRNA expression was significantly increased by the stimulation of IL-1β and TNF-α dose dependently (Fig. 6). However, Sn expression was not induced by hyperglycemic

condition or stimulation with IL-4, and TGF-β1 (Fig. 6). Furthermore, we confirmed that mRNA expression of IL-1β and TNF-α is upregulated in diabetic glomeruli (Fig. 7). These data indicate that inflammatory cytokines, *i.e.* IL-1β and TNF-α, but not hyperglycemia stimulate the expression of Sn in macrophages.

Hyperglycemia causes glomerular hyperfiltration and glomerular hypertrophy (28-29). Increased intraglomerular pressure caused by hyperfiltration may activate the mesangial cells because phenotypic change of mesangial cells is brought about by mechanical stress in vitro (30). Growth factors including insulin like growth factor-1 (IGF-1) and platelet derived growth factor (PDGF) are known to be involved in glomerular hypertrophy (31-33) and may play an important role in the phenotypic change of mesangial cells. Growth factors such as IGF-1 and PDGF are synthesized by macrophages, mesangial cells and others (34-35). From these findings, phenotypic change of mesangial cells might be caused by macrophages derived growth factors. Although our current study showed that the phenotypic change of mesangial cells was in parallel with increase of Sn-positive macrophages, the mechanism of interaction between macrophages and mesangial cells remains unclear and further studies are needed.

In conclusion, the current results suggest that macrophages are activated in diabetic glomeruli and Sn-positive activated macrophages may contribute to the progression of diabetic nephropathy through activation of mesangial cells.

Acknowledgments

This study was supported in part by Grant-in-Aid for Scientific Research (C) to K. Shikata (21591031), Grant-in-Aid for Young Scientists (B) to D. Ogawa (23790942)

from the Ministry of Education, Culture, Sports, Science and Technology and by Grant-in-Aid for Diabetic Nephropathy from the Ministry of Health, Labour and Welfare of Japan. This work has received support from the Takeda Science Foundation, the Naito Foundation, the Japan Foundation for Applied Enzymology, and the Ryobi Teien Memory Foundation.

Reprint requests to Kenichi Shikata, M.D., Ph.D. and Daisuke Ogawa, M.D., Ph.D., Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan.
E-mail: shikata@md.okayama-u.ac.jp, daiogawa@md.okayama-u.ac.jp

References

1. Furuta T, Saito T, Ootaka T, Soma J, Obara K, Abe K, et al. The role of macrophages in diabetic glomerulosclerosis. Am J Kidney Dis. 1993 May;21(5):480-5.

2. Sugimoto H, Shikata K, Hirata K, Akiyama K, Matsuda M, Kushiro M, et al. Increased expression of intercellular adhesion molecule-1 (ICAM-1) in diabetic rat glomeruli: glomerular hyperfiltration is a potential mechanism of ICAM-1 upregulation. Diabetes. 1997 Dec;46(12):2075-81.

3. Okada S, Shikata K, Matsuda M, Ogawa D, Usui H, Kido Y, et al. Intercellular adhesion molecule-1-deficient mice are resistant against renal injury after induction of diabetes. Diabetes. 2003 Oct;52(10):2586-93.

4. Crocker PR, Gordon S. Properties and distribution of a lectin-like hemagglutinin differentially expressed by murine stromal tissue macrophages. J Exp Med. 1986 Dec 1;164(6):1862-75.

5. Crocker PR, Gordon S. Mouse macrophage hemagglutinin (sheep erythrocyte receptor) with specificity for sialylated glycoconjugates characterized by a monoclonal antibody. J Exp Med. 1989 Apr 1;169(4):1333-46.

6. Crocker PR, Hill M, Gordon S. Regulation of a murine macrophage haemagglutinin (sheep erythrocyte receptor) by a species-restricted serum factor. Immunology. 1988 Dec;65(4):515-22.

7. van den Berg TK, van Die I, de Lavalette CR, Dopp EA, Smit LD, van der Meide PH, et al. Regulation of sialoadhesin expression on rat macrophages. Induction by glucocorticoids and enhancement by IFN-beta, IFN-gamma, IL-4, and lipopolysaccharide. J Immunol. 1996 Oct 1;157(7):3130-8.

8. Steiniger B, Barth P, Herbst B, Hartnell A, Crocker PR. The species-specific structure of microanatomical compartments in the human spleen: strongly sialoadhesin-positive macrophages occur in the perifollicular zone, but not in the marginal zone. Immunology. 1997 Oct;92(2):307-16.

9. Ito Y, Kawachi H, Morioka Y, Nakatsue T, Koike H, Ikezumi Y, et al. Fractalkine expression and the recruitment of CX3CR1+ cells in the prolonged mesangial proliferative glomerulonephritis. Kidney Int. 2002 Jun;61(6):2044-57.

10. Lan HY, Nikolic-Paterson DJ, Mu W, Atkins RC. Local macrophage proliferation in the progression of glomerular and tubulointerstitial injury in rat anti-GBM glomerulonephritis. Kidney Int. 1995 Sep;48(3):753-60.

11. Lai PC, Cook HT, Smith J, Keith JC, Jr., Pusey CD, Tam FW. Interleukin-11 attenuates nephrotoxic nephritis in Wistar Kyoto rats. J Am Soc Nephrol. 2001 Nov;12(11):2310-20.

12. Ikezumi Y, Kanno K, Karasawa T, Han GD, Ito Y, Koike H, et al. The role of lymphocytes in the experimental progressive glomerulonephritis. Kidney Int. 2004 Sep;66(3):1036-48.

13. Dijkstra CD, Dopp EA, Joling P, Kraal G. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology. 1985 Mar;54(3):589-99.

14. Mauer SM, Steffes MW, Ellis EN, Sutherland DE, Brown DM, Goetz FC. Structural-functional relationships in diabetic nephropathy. J Clin Invest. 1984 Oct;74(4):1143-55.

15. Osterby R, Parving HH, Hommel E, Jorgensen HE, Lokkegaard H. Glomerular structure and function in diabetic nephropathy. Early to advanced stages. Diabetes. 1990 Sep;39(9):1057-63.

16. Makino H, Yamasaki Y, Hironaka K, Ota Z. Glomerular extracellular matrices in rat diabetic glomerulopathy by scanning electron microscopy. Virchows Arch B Cell Pathol Incl Mol Pathol. 1992;62(1):19-24.

17. Makino H, Yamasaki Y, Haramoto T, Shikata K, Hironaka K, Ota Z, et al. Ultrastructural changes of extracellular matrices in diabetic nephropathy revealed by

high resolution scanning and immunoelectron microscopy. Lab Invest. 1993 Jan;68(1):45-55.

18. Abrass CK, Peterson CV, Raugi GJ. Phenotypic expression of collagen types in mesangial matrix of diabetic and nondiabetic rats. Diabetes. 1988 Dec;37(12):1695-702.

19. Makino H, Shikata K, Wieslander J, Wada J, Kashihara N, Yoshioka K, et al. Localization of fibril/microfibril and basement membrane collagens in diabetic glomerulosclerosis in type 2 diabetes. Diabet Med. 1994 Apr;11(3):304-11.

20. Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzi P, et al. Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial cell proliferation. J Clin Invest. 1991 Mar;87(3):847-58.

21. Alpers CE, Hudkins KL, Gown AM, Johnson RJ. Enhanced expression of "muscle-specific" actin in glomerulonephritis. Kidney Int. 1992 May;41(5):1134-42.

22. Kodera R, Shikata K, Kataoka HU, Takatsuka T, Miyamoto S, Sasaki M, et al. Glucagon-like peptide-1 receptor agonist ameliorates renal injury through its anti-inflammatory action without lowering blood glucose level in a rat model of type 1 diabetes. Diabetologia. 2011 Apr;54(4):965-78.

23. Takeya M, Hsiao L, Takahashi K. A new monoclonal antibody, TRPM-3, binds specifically to certain rat macrophage populations: immunohistochemical and immunoelectron microscopic analysis. J Leukoc Biol. 1987 Mar;41(3):187-95.

24. Crocker PR, Mucklow S, Bouckson V, McWilliam A, Willis AC, Gordon S, et al. Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. EMBO J. 1994 Oct 3;13(19):4490-503.

25. Hartnell A, Steel J, Turley H, Jones M, Jackson DG, Crocker PR. Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. Blood. 2001 Jan 1;97(1):288-96.

26. Crocker PR, Varki A. Siglecs, sialic acids and innate immunity. Trends Immunol. 2001 Jun;22(6):337-42.

27. Crocker PR, Hartnell A, Munday J, Nath D. The potential role of sialoadhesin as a macrophage recognition molecule in health and disease. Glycoconj J. 1997 Aug;14(5):601-9.

28. Wiseman MJ, Viberti GC, Keen H. Threshold effect of plasma glucose in the glomerular hyperfiltration of diabetes. Nephron. 1984;38(4):257-60.

29. Osterby R, Gundersen HJ. Fast accumulation of basement membrane material and the rate of morphological changes in acute experimental diabetic glomerular hypertrophy. Diabetologia. 1980 Jun;18(6):493-500.

30. Harris RC, Haralson MA, Badr KF. Continuous stretch-relaxation in culture alters rat mesangial cell morphology, growth characteristics, and metabolic activity. Lab Invest. 1992 May;66(5):548-54.

31. Miyatake N, Shikata K, Wada J, Sugimoto H, Takahashi S, Makino H. Differential distribution of insulin-like growth factor-1 and insulin-like growth factor binding proteins in experimental diabetic rat kidney. Nephron. 1999;81(3):317-23.

32. Floege J, Topley N, Hoppe J, Barrett TB, Resch K. Mitogenic effect of platelet-derived growth factor in human glomerular mesangial cells: modulation and/or suppression by inflammatory cytokines. Clin Exp Immunol. 1991 Nov;86(2):334-41.

33. Fukui M, Nakamura T, Ebihara I, Makita Y, Osada S, Tomino Y, et al. Effects of enalapril on endothelin-1 and growth factor gene expression in diabetic rat glomeruli. J Lab Clin Med. 1994 May;123(5):763-8.

34. Shimokado K, Raines EW, Madtes DK, Barrett TB, Benditt EP, Ross R. A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. Cell. 1985 Nov;43(1):277-86.

35. Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. Cell. 1986 Jul 18;46(2):155-69.

Figure Legends

Figure. 1 Identification of macrophages in diabetic rat glomeruli. Indirect immunofluorescent micrographs from diabetic rat glomeruli 24 weeks after STZ injection, stained with anti-ED1 (pan-macrophages) (a), anti-ED3 (sialoadhesin (Sn)-positive macrophages) (d), anti-OX-6 (MHC class II positive cells) (b, e), anti-ED1 + anti-OX-6 (c), anti-ED3 + anti-OX-6 (f). magnification ×200. Intraglomerular infiltration by pan-macrophages (ED1) (g), resident macrophages (ED2) (h), and Sn-positive macrophages (ED3) (i) in diabetic rats (○), in control rats (●) at 0, 1, 4, 12, and 24 weeks, and insulin treated diabetic rats (□) at 4 weeks. A straight line represents ED1, ED2, and ED3 and a broken line represents ED1, ED2, and ED3 plus OX-6. Data are means±SEM of 50 glomeruli, respectively. *$p < 0.05$ vs. control, ** $p < 0.01$ vs. control, ***$p < 0.005$ vs. control.

Figure. 2 Identification of ICAM-1 in diabetic glomeruli. Indirect immunofluorescent micrographs from control rats at 24 weeks (a) and diabetic rats at 24 weeks after STZ injection (b), stained with ICAM-1, magnification ×200. c: Intraglomerular ICAM-1 expression in control rats (●), diabetic rats (○) at 0, 1, 4, 12, and 24 weeks, and insulin treated diabetic rats (□) at 4 weeks. ***$p < 0.005$ vs. control.

Figure. 3 Identification of α-smooth muscle actin (SMA) in diabetic glomeruli. Immunoperoxidase staining for α-SMA in control rats at 24 weeks (a) and diabetic rats at 24 weeks after STZ injection (b), magnification ×200. c: Intraglomerular α-smooth muscle actin expression in control rats (●), diabetic rats (○) at 0, 1, 4, 12, and 24

weeks, and insulin treated diabetic rats (☐) at 4 weeks. *p < 0.05 vs. control, ** p < 0.01 vs. control.

Figure. 4 Identification of type IV collagen in diabetic glomeruli. Indirect immunofluorescent micrographs from control rats at 24 weeks (a) and diabetic rats at 24 weeks after STZ injection (b), stained with type IV collagen, magnification ×200. c: Intraglomerular type IV collagen expression in control rats (●), diabetic rats (○) at 0, 1, 4, 12, and 24 weeks, and insulin treated diabetic rats (☐) at 4 weeks. *p < 0.05 vs. control, ** p < 0.01 vs. control, ***p < 0.005 vs. control.

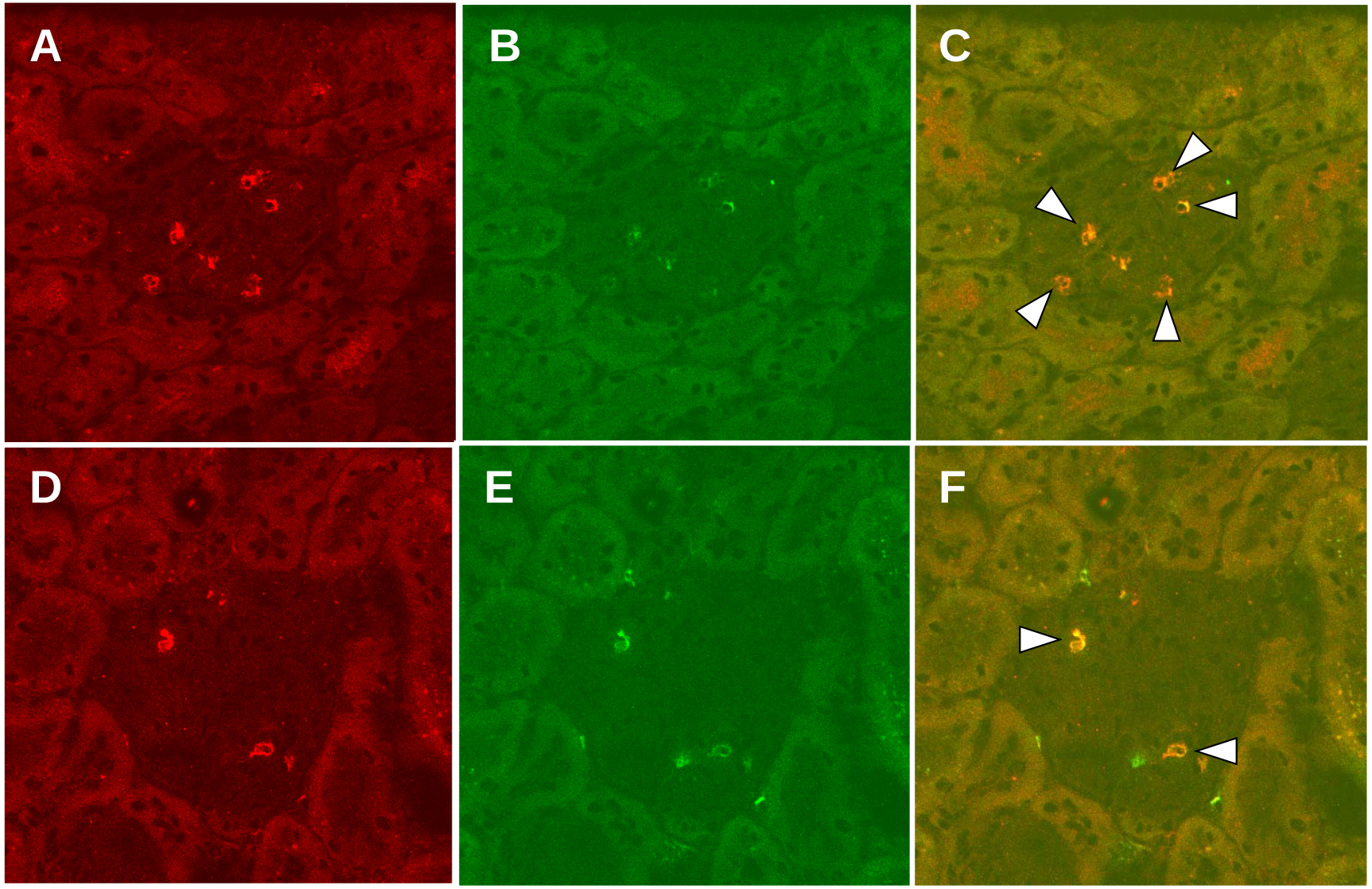
Figure. 5 Identification and quantification of Periodic acid-methenamine-silver (PAM)-positive area in diabetic glomeruli. PAM staining of kidney sections in control rats at 24 weeks (a) and diabetic rats at 24 weeks after STZ injection (b), magnification ×200. c: Mesangial matrix area is defined as PAM-positive area in the tuft area in control rats (●), diabetic rats (○) at 0, 1, 4, 12, and 24 weeks, and insulin treated diabetic rats (☐) at 4 weeks. *** p < 0.005 vs. control.

Figure. 6 Transcriptional regulation of sialoadhesin (Sn) expression on THP-1 cells. Sn mRNA expression after 24h exposure to various cytokines or hyperglycemic condition was determined by quantitative real-time RT-PCR. HG: hyperglycemic condition. HO: hyperosmotic control. n=3 per each group. Data are Mean±SE. *p<0.05 compared with vehicle (ANOVA).

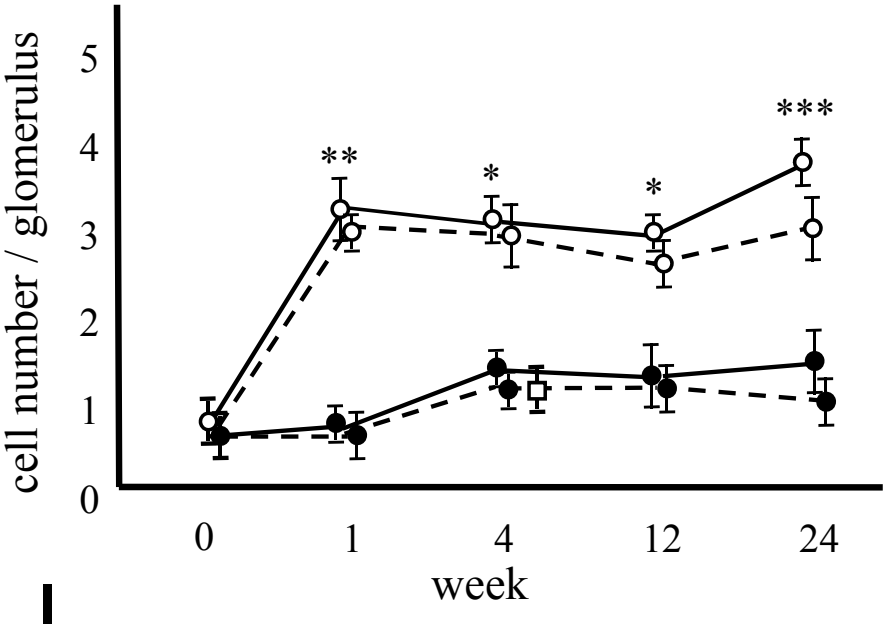
Figure. 7 Cytokine expressions in rat kidney. mRNA expression of IL-1β and

TNF-α was determined by quantitative real-time RT-PCR. mRNA expression of IL-1β and TNF-α is upregulated in diabetic glomeruli. n=3 per each group. Data are Mean±SE. *p<0.05 compared with vehicle (ANOVA).

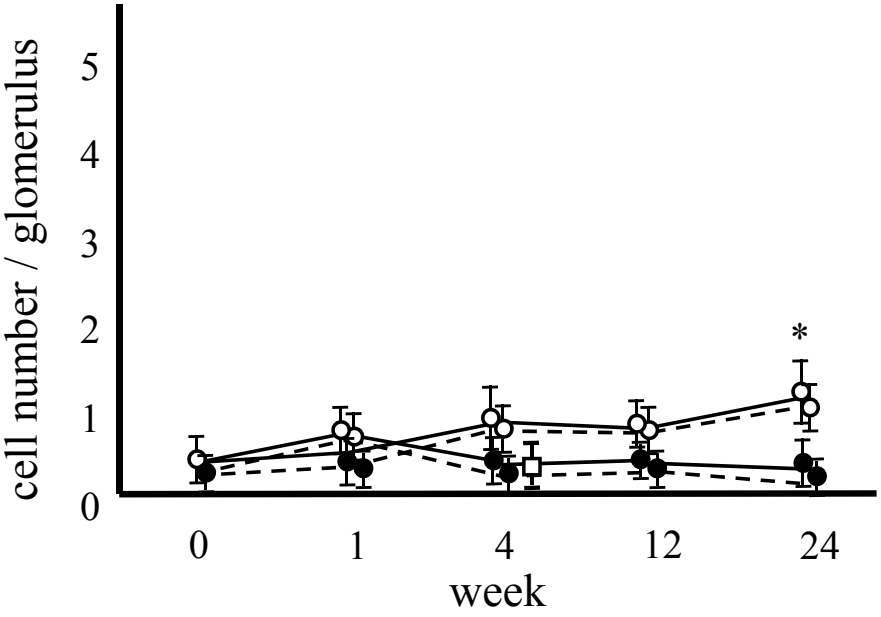
Nagase R. et al Figure 1



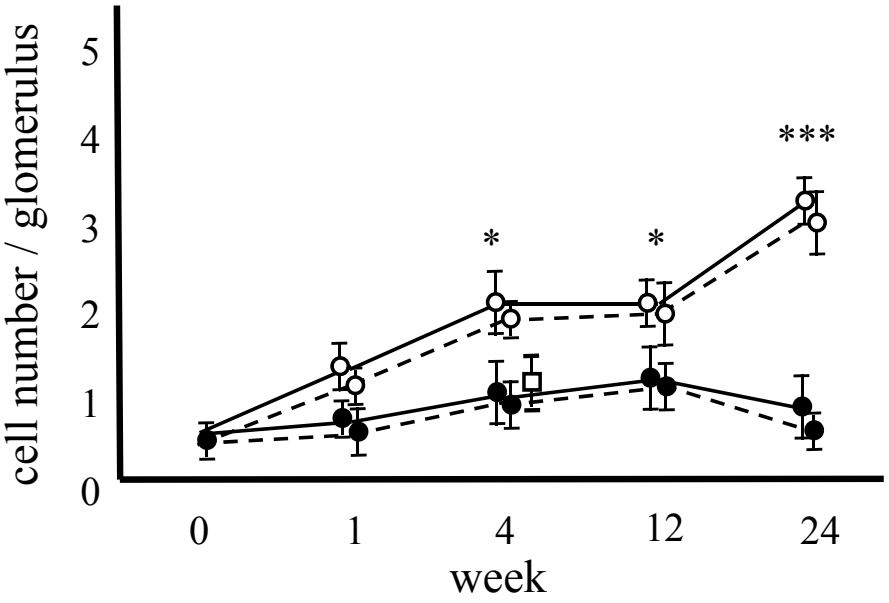
G


cell number / glomerulus
0
1
2
3
4
5
**
*
*
***
0
1
4
12
24
week

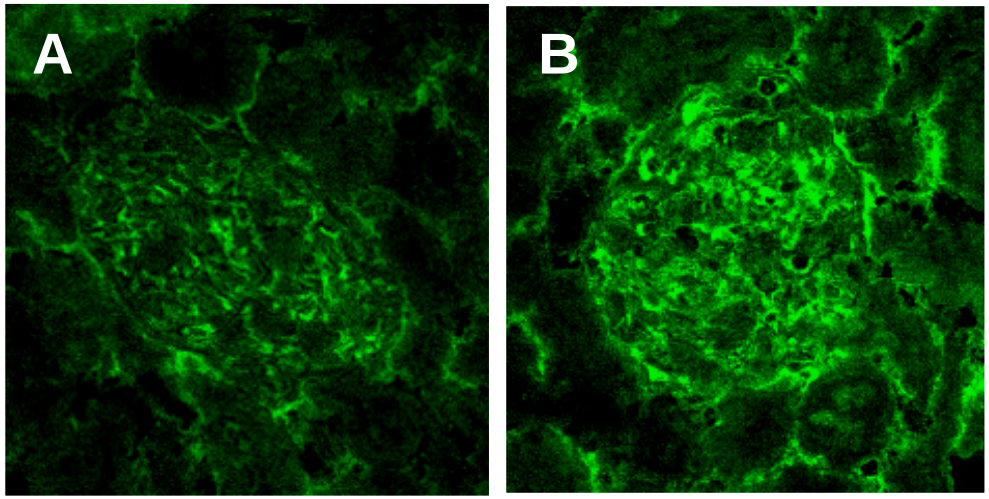
H

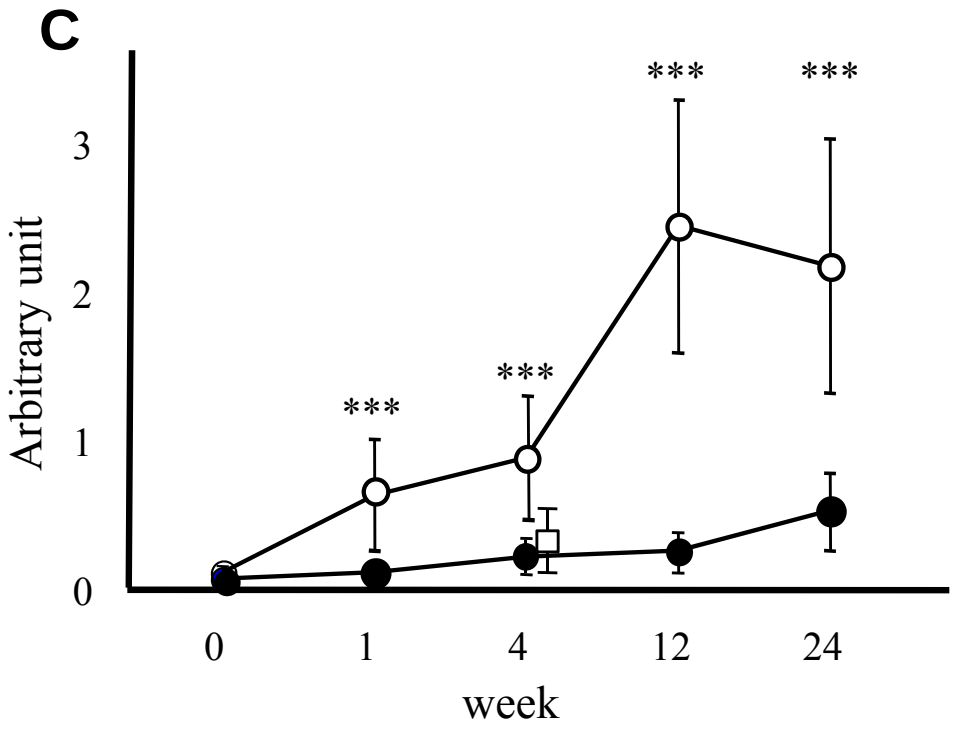

cell number / glomerulus
0
1
2
3
4
5
*
0
1
4
12
24
week

I

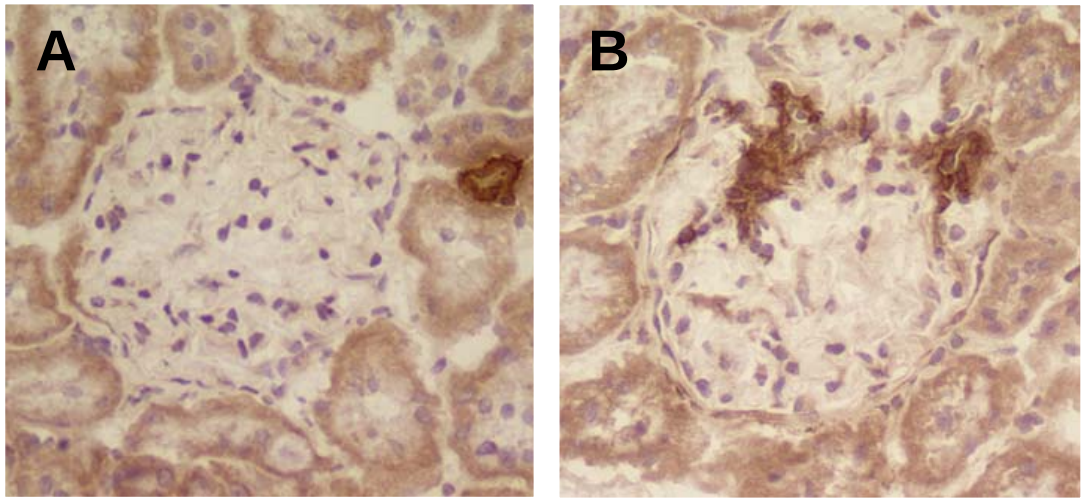

cell number / glomerulus
0
1
2
3
4
5
*
*
***
0
1
4
12
24
week

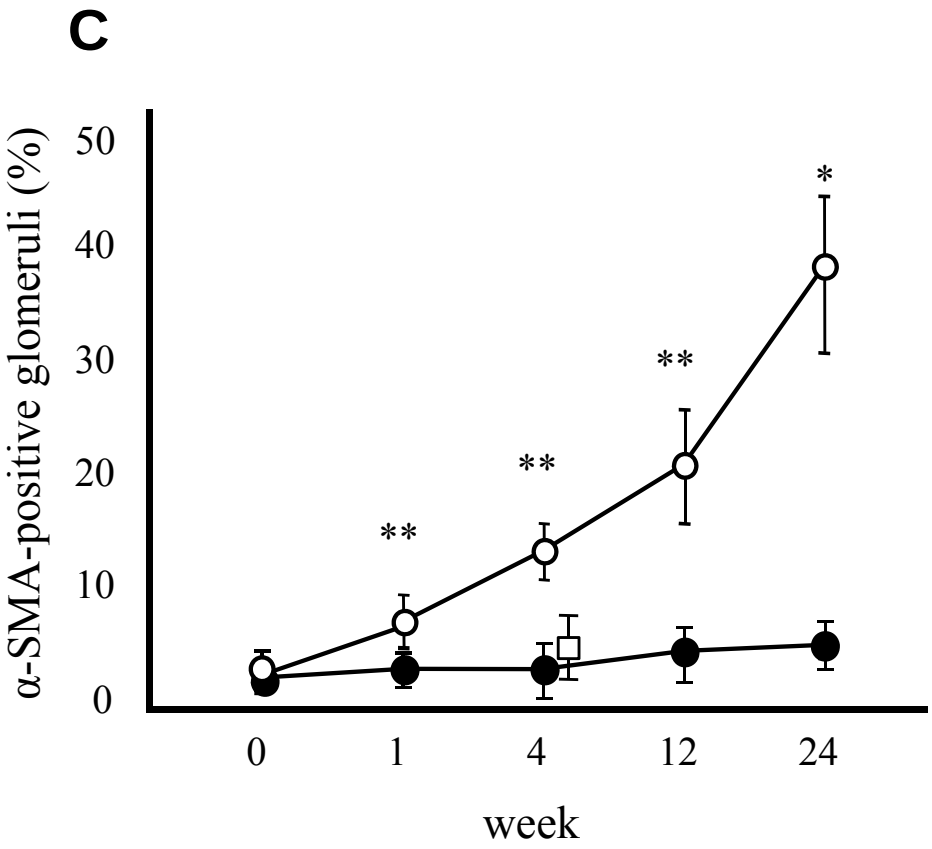
Nagase R. et al Figure 2



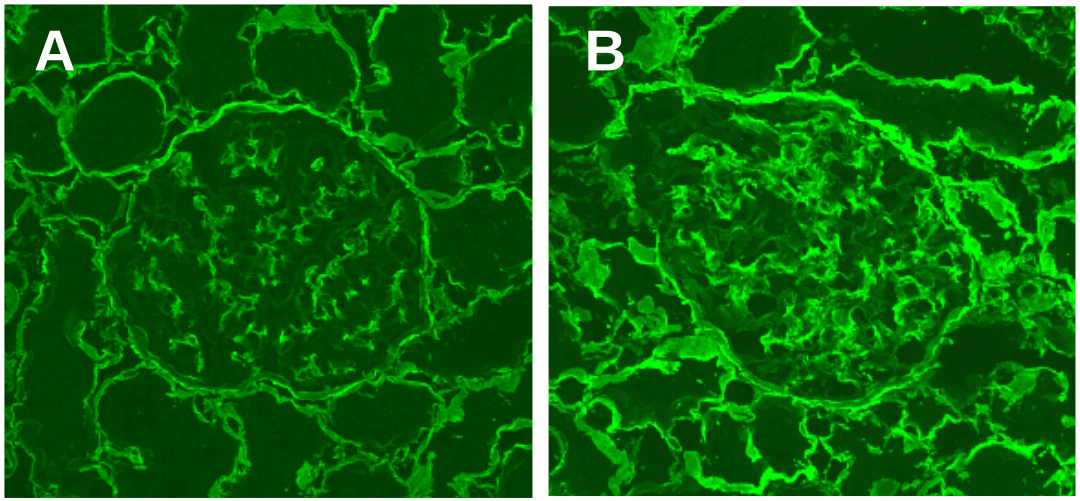


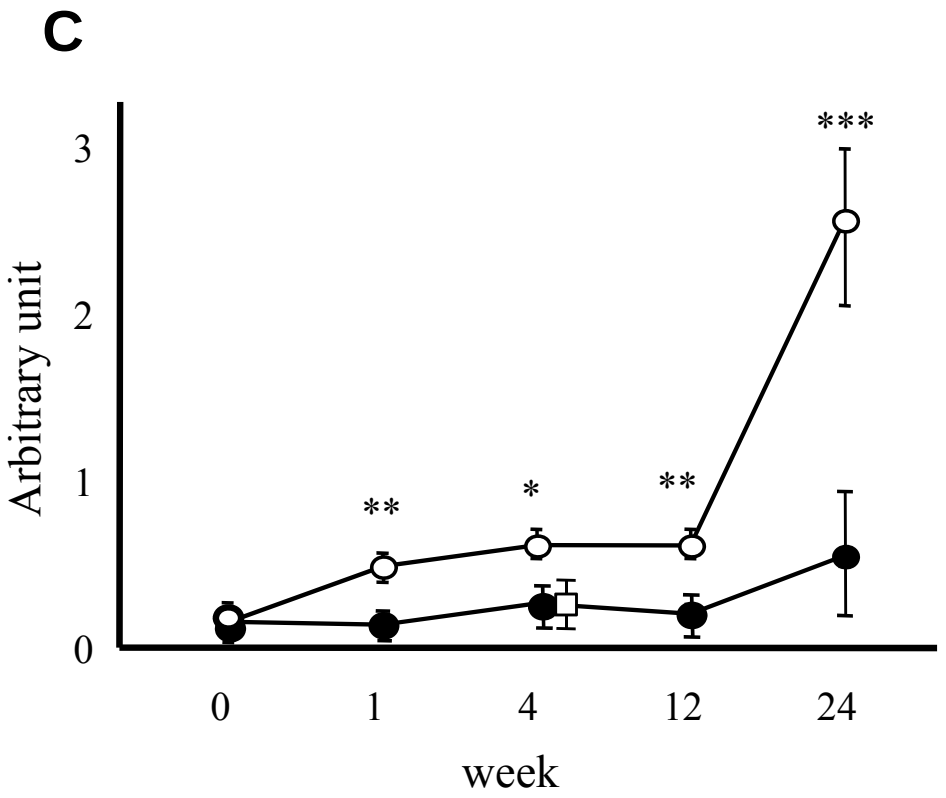
Nagase R. et al Figure 3



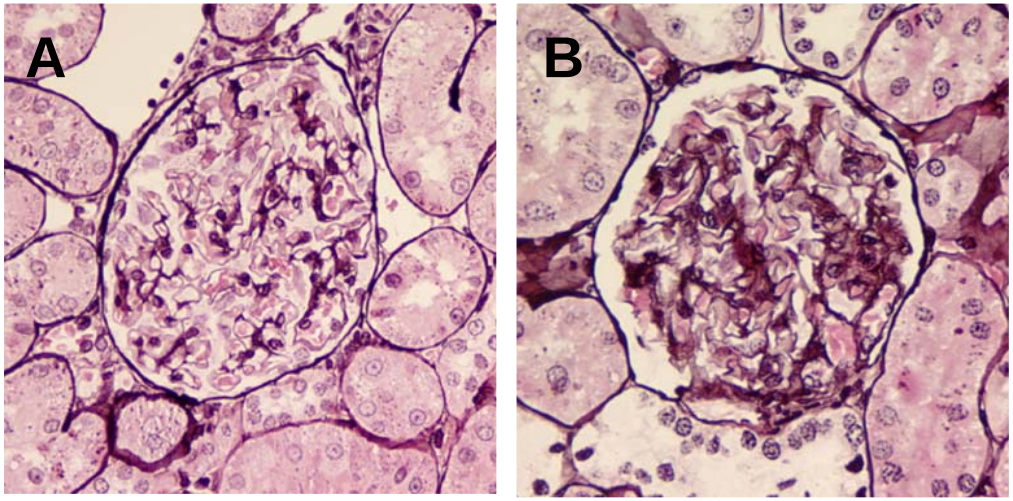


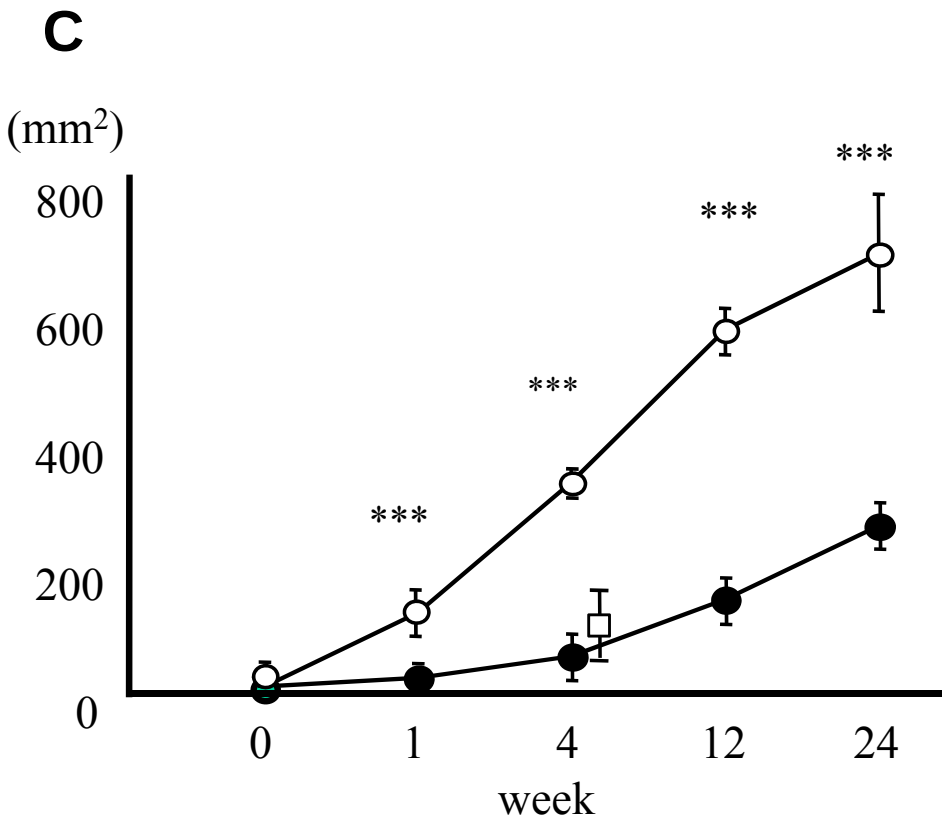
Nagase R. et al Figure 4



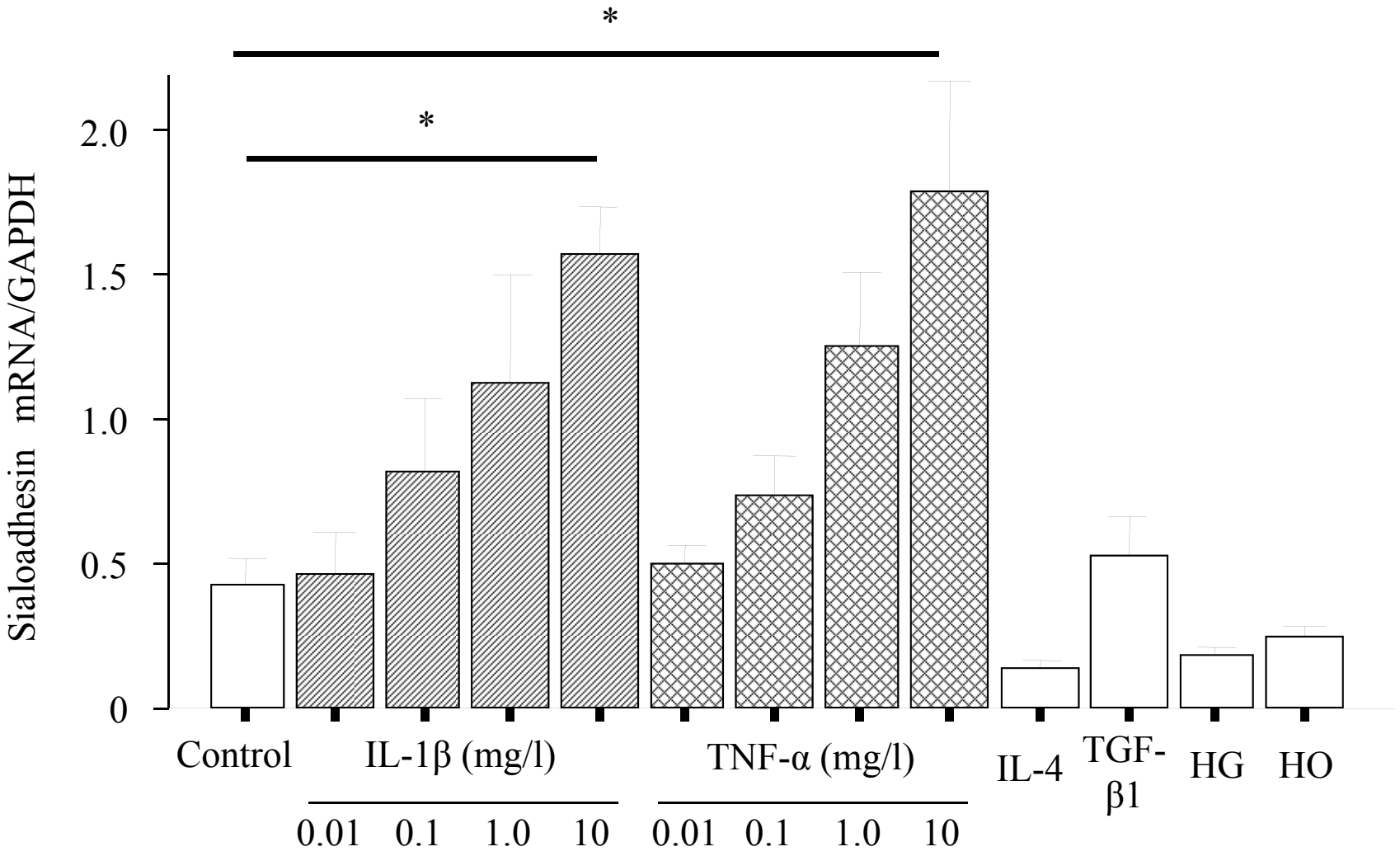


Nagase R. et al Figure 5





Nagase R. et al Figure 6



Nagase R. et al Figure 7