Gamma linolenic acid exerts anti-inflammatory and anti-fibrotic effects in diabetic nephropathy

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Gamma linolenic acid exerts anti-inflammatory and anti-fibrotic effects in diabetic nephropathy

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<TABLE OF CONTENTS>

ABSTRACT·····1

I. INTRODUCTION·····4

II. MATERIALS AND METHODS·····6

1. Animals·····6
2. Cell culture·····7
3. Total RNA extraction·····8
4. Reverse transcription·····8
5. Real-time polymerase chain reaction (Real-time PCR) ·····9
6. Western blot analysis·····10
7. Measurement of MCP-1 by ELISA·····11
8. Pathology·····11
9. Statistical analysis·····13

III. RESULTS

1. Animal studies·····13
   A. Animal data·····13
   B. Effect of GLA on renal cortical MCP-1 mRNA and protein expression·····14
   C. Effect of GLA on renal cortical ICAM-1 mRNA and protein expression·····16
   D. Effect of GLA on renal cortical fibronectin mRNA and protein expression·····16
   E. Effect of GLA on macrophage accumulation·····17
   F. Effect of GLA on glomerular and tubulointerstitial fibrosis·····17

2. Cell culture studies·····22
   A. Effect of GLA on MCP-1 mRNA and protein expression·····22
   B. Effect of GLA on ICAM-1 mRNA and protein expression·····25
   C. Effect of GLA on fibronectin mRNA and protein expression·····25

IV. DISCUSSION·····29

V. CONCLUSION·····34

REFERENCES·····36

ABSTRACT (IN KOREAN) ·····43

LIST OF FIGURES

Figure 1. Renal MCP-1, ICAM-1, and fibronectin mRNA/18s rRNA ratios in C, C+GLA, DM, and DM+GLA rats·····15

Figure 2. Renal MCP-1 protein levels in C, C+GLA, DM, and DM+GLA rats ·····15

Figure 3. Renal ICAM-1 and fibronectin protein expression in C, C+GLA, DM, and DM+GLA rats ·····18

Figure 4. Immunohistochemical staining for glomerular ICAM-1, fibronectin, and ED-1 in C, C+GLA, DM, and DM+GLA rats ····· 19

Figure 5. Immunohistochemical staining for tubulointerstitial ICAM-1, fibronectin, and ED-1 in C, C+GLA, DM, and DM+GLA rats. ·····20

Figure6. Masson's trichrome staining for glomerular and tubulointerstitial fibrosis in C, C+GLA, DM, and DM+GLA rats ·····21

Figure 7. MCP-1 mRNA/18s rRNA ratios in mesangial cells and NRK-52E cells exposed to NG, NG+M, NG+GLA, HG, and HG+GLA medium·····23

Figure 8. MCP-1 levels in conditioned culture media from mesangial cells and NRK-52E cells exposed to NG, NG+M, NG+GLA, HG, and HG+GLA medium ·····24

Figure 9. ICAM-1 mRNA/18s rRNA ratios in mesangial cells and NRK-52E cells exposed to NG, NG+M, NG+GLA, HG, and HG+GLA medium·····26
Figure 10. A representative Western blots of ICAM-1 and fibronectin in cultured mesangial cells and NRK-52E cells····27
Figure 11. Fibronectin mRNA/18s rRNA ratios in mesangial cells cells and NRK-52E cells exposed to NG, NG+M, NG+GLA, HG, and HG+GLA medium·····28

LIST OF TABLE

Table 1. Animal data of the four groups·····14

<ABSTRACT>

Gamma linolenic acid exerts anti-inflammatory and anti-fibrotic effects in diabetic nephropathy

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Background: Accumulating evidence suggests that an inflammatory mechanism contributes to the development and progression of diabetic nephropathy. Gamma linolenic acid (GLA), a member of polyunsaturated fatty acids (PUFAs), has been reported to have an anti-inflammatory effect by generating modulatory molecules for inflammatory responses. In addition, previous studies have demonstrated that GLA abrogates rheumatologic diseases and diabetic neuropathy via an anti-inflammatory mechanism. However, the effect of GLA on diabetic nephropathy has been largely unexplored.

Purpose: This study was undertaken to investigate the effects of GLA on inflammation and extracellular matrix (ECM) synthesis in mesangial and tubular epithelial cells under diabetic conditions.

Methods: *In vivo*, 32 Sprague-Dawley rats were injected either with diluent [n=16, control(C)] or streptozotocin intraperitoneally [n=16, diabetes(DM)], and 8 rats from control and diabetic groups were treated with evening primrose oil by gavage (450 mg/kg/day) for 3 months. *In vitro*, rat mesangial cells and NRK-52E cells were exposed to medium containing 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), and 30 mM glucose (HG) with or without GLA (10 or 100 μM). Real-time PCR and Western blot were performed for intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), and fibronectin (FN) mRNA and protein expression, respectively. Immunohistochemical staining for ICAM-1 and FN, and Masson's trichrome staining with renal tissue were also performed.

Results: Twenty four-hour urinary albumin excretion was significantly increased in DM compared to C rats ($p<0.05$), and GLA treatment significantly reduced albuminuria in DM rats ($p<0.05$). ICAM-1, MCP-1, and FN mRNA and protein expression were significantly increased in DM compared to C kidney, and these increases were significantly abrogated by GLA treatment. In addition, the extent of glomerular and tubulointerstitial fibrosis assessed by Masson's trichrome staining was significantly greater in DM relative to C kidney ($p<0.005$), and this change was significantly ameliorated by the administration of GLA ($p<0.01$). *In vitro*, GLA

significantly inhibited the increases in MCP-1 mRNA expression and protein levels under high glucose conditions in HG-stimulated mesangial and tubular epithelial cells ($p<0.05$). ICAM-1 and FN expression showed a similar pattern to the expression of MCP-1.

Conclusion: GLA attenuates not only inflammation via inhibiting enhanced MCP-1 and ICAM-1 expression but also ECM accumulation in diabetic nephropathy.

Key words : GLA, diabetic nephropathy, MCP-1, ICAM-1, fibronectin

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I. INTRODUCTION

Diabetic nephropathy, the leading cause of end-stage renal disease (ESRD) worldwide, is characterized pathologically by cellular hypertrophy and increased extracellular matrix (ECM) accumulation[1]. The ECM accumulation in diabetic nephropathy results in mesangial expansion, tubulointerstitial fibrosis, and irreversible deterioration of renal function[2]. Even though previous studies have shown that ECM accumulation under diabetic conditions are attributed to hyperglycemia *per se*, advanced glycation end-products, hemodynamic changes, and local growth factors such as angiotensin II (AII) and transforming growth factor (TGF)-β1[3], the precise molecular and cellular mechanisms responsible for this still remain to be resolved.

Recently, accumulating evidence suggests that inflammatory process also plays an important role in the pathogenesis of diabetic nephropathy[4,5]. Infiltration of inflammatory cells in the glomeruli and renal tubulointerstitium is commonly seen

in both human diabetic patients and experimental diabetic animals[6-8]. In addition, intracellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1), which mediates the recruitment and infiltration of monocytes/macrophages, are demonstrated to be involved in the pathogenesis of diabetic nephropathy[9-11]. Based on these findings, modulation of the inflammatory process is considered to be a potential means of preventing the development and progression of diabetic nephropathy[12,13], and some immunosuppressive agents and anti-inflammatory drugs are found to be beneficial in diabetic nephropathy. Nevertheless, chronic use of these drugs in a clinical field is not appropriate due to many systemic side effects. Therefore, other safe agents for chronic treatment in diabetic nephropathy are inevitably needed.

Polyunsaturated fatty acids (PUFAs), which exist in high concentrations in cell membranes as structural phospholipids, are essential for cell integrity and viability[14-16]. There are two classes of PUFAs; ω-3 and ω-6, designated according to their carbon ring structure[17-19]. ɣ-linolenic acid (GLA) is a member of ω-6 PUFA, is produced from linoleic acid by the enzyme of δ-6 desaturase, and is elongated to dihomogamma linolenic acid (DGLA)[16,20]. In a previous study, GLA was shown to abrogate renal fibrosis in a 5/6 nephrectomy model[21], and other investigations demonstrated that GLA treatment improved autoimmune diseases and diabetic neuropathy via an anti-inflammatory mechanism[22,23]. As aforementioned, since inflammatory process is also involved in the pathogenesis of diabetic nephropathy, there is a possibility that GLA may ameliorate diabetic nephropathy,

but it has never been explored.

In this study, therefore, I investigated the effects of GLA in experimental diabetic kidney and in high glucose-stimulated mesangial cells and tubular epithelial cells in terms of inflammation and ECM synthesis.

II. MATERIALS AND METHODS

1. Animals

All animal studies were conducted under an approved protocol. Rats weighing 250-280 g were injected with either diluent [n=16, Control (C)] or 65 mg/kg streptozotocin (STZ) intraperitoneally [n=16, Diabetes (DM)]. Diabetes was confirmed by tail vein blood glucose levels on the third post-injection day. After confirming diabetes, eight rats from C and DM groups were treated with 450 mg/kg/day of evening primrose oil (EPO, a generous gift from Dalim Biotech, Seoul, Korea) by gavage (C+GLA or DM+GLA) for 3 months. EPO contains 8-10% GLA and the amount of EPO used in this study gives an approximate dose of 40 mg/kg/day of GLA. Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 3-month study period.

Body weights and serum glucose levels were checked monthly, and kidney weights and 24-hour urinary albumin excretion at the time of sacrifice. Blood glucose was measured by a glucometer and 24-hour urinary albumin excretion was

determined by enzyme-linked immunosorbent assay (ELISA) (Nephrat II, Exocell, Inc., Philadelphia, PA, USA).

2. Cell culture

Primary culture of glomerular mesangial cells were done as previously described[24]. Identification of mesangial cells was performed by their characteristic stellate appearance in culture and confirmed by immunofluorescent microscopy for the presence of actin, myosin, and Thy-1 antigen and the absence of factor VIII and cytokeratin (Synbiotics, San Diego, CA, USA). Mesangial cells and NRK-52E cells, immortalized rat tubular epithelial cells, were maintained in RPMI 1640 and DMEM medium, respectively, supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 26 mM $NaHCO_3$, and were grown at 37℃ in humidified 5% CO_2 in air. Subconfluent mesangial cells and NRK-52E cells were serum restricted for 24 hours, after which the medium was replaced by serum-free medium containing 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), or 30 mM glucose (HG) with or without 10 or 100 μM GLA (Sigma Chemical Co., St Louis, MO, USA). At 24 hours after the media change, cells were harvested and the conditioned culture media were collected.

3. Total RNA extraction

Total RNA from the renal cortical tissue was extracted as previously described[24]. Briefly, 100 μl of RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA) was added to the renal cortical tissues, which were lysed by freezing and thawing three times. Another 700 μl of RNA STAT-60 reagent was then added and the mixture was vortexed and stored for 5 minutes at room temperature. Next, 160 μl of chloroform was added and the mixture was shaken vigorously for 30 seconds. After 3 minutes, the mixture was centrifuged at 12,000 X g for 15 minutes at 4℃ and the upper aqueous phase containing the extracted RNA was transferred to a new tube. RNA was precipitated from the aqueous phase by adding 400 μl of isopropanol and then pelleted by centrifugation at 12,000 X g for 30 minutes at 4℃. The RNA precipitate was washed with 70% ice-ethanol, dried using a Speed Vac, and dissolved in DEPC-treated distilled water. RNA yield and quality were assessed based on spectrophotometric measurements at wavelengths of 260 and 280 nm. Total RNA from mesangial cells and NRK-52E cells was extracted similarly.

4. Reverse transcription

First strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). Two μg of total RNA extracted from the renal cortex and cultured cells were reverse transcribed using 10 μM random hexanucleotide primer, 1 mM dNTP, 8 mM $MgCl_2$, 30 mM KCl, 50 mM Tris-HCl, pH 8.5, 0.2 mM dithiothreithol, 25 U RNase inhibitor, and 40 U

AMV reverse transcriptase. The mixture was incubated at 30℃ for 10 minutes and 42℃ for 1 hour followed by inactivation of the enzyme at 99℃ for 5 minutes.

5. Real-time polymerase chain reaction (Real-time PCR)

The primers used for ICAM-1, MCP-1, fibronectin, and 18s amplification were as follows: ICAM-1, sense 5'-AGGTA TCCATCCATCCCAC-3', antisense 5'-GCCGAGGTTCTCGTCTTC-3'; MCP-1, sense 5'-TCTCTTCCTCCACCACTAT GCA-3', antisense 5'-GGCTGAGACAGCACGTGGAT-3'; fibronectin, sense 5'-TGACAACTGCCGTAGACCTGG-3', antisense 5'-TACTGGTTGTAGGTGTGG CCG-3'; and 18s, sense 5'-AGTCCCTGCCCTTTGT ACACA-3', antisense 5'-GATCCGAGGGCCTCACTAAAC-3'. cDNAs from 25 ng RNA of the renal cortical tissue or cultured cells per reaction tube were used for amplification.

Using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), PCR was performed with a total volume of 20 μl in each well, containing 10 μl of SYBR Green® PCR Master Mix (Applied Biosystems), 5 μl of cDNA, and 5 pM sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes. The PCR conditions were as follows: 35 cycles of denaturation at 94.5℃ for 30 sec, annealing at 60℃ for 30 sec, and extension at 72℃ for 1 minute. Initial heating at 95℃ for 9 minutes and final extension at 72℃ for 7 minutes were performed for all PCRs.

After real-time PCR, the temperature was increased from 60 to 95℃ at a rate of 2℃/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative C_T method with $2^{-\Delta\Delta T}$. The results are given as relative expression of ICAM-1, MCP-1, and fibronectin normalized to the expression of the 18s housekeeping gene.

6. Western blot analysis

The renal cortical tissue and cultured cells harvested from plates were lysed in sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol), treated with Laemmli sample buffer, heated at 100℃ for 5 minutes, and electrophoresed in an 8% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA), and the membrane was then incubated in blocking buffer A (1 x PBS, 0.1% Tween-20, and 8% nonfat milk) at room temperature for 1 hour, followed by an overnight incubation at 4℃ in a 1:1000 dilution of polyclonal antibodies to ICAM-1 (R&D systems, Minneapolis, MN, USA), fibronectin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or β-actin (Sigma Chemical Co.). The membrane was then washed once for 15 minutes and twice for 5 minutes in 1 x PBS with 0.1% Tween-20. Next, the membrane was incubated in buffer A containing a 1:1000 dilution of horseradish peroxidase-linked donkey anti-goat IgG

(Amersham Life Science, Inc., Arlington Heights, IL, USA). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Inc.). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

7. Measurement of MCP-1 by ELISA

The levels of MCP-1 in the renal cortical tissue and culture media were determined using a commercial ELISA kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. The kit for rat MCP-1 was species-specific and sensitive up to 750 pg/ml. All the concentrations of MCP-1 were normalized to the total protein amount.

8. Pathology

For immunohistochemical staining, slices of the kidney were snap-frozen in optimal cutting temperature (OCT) solution and 4 μm sections of tissues were utilized. Slides were fixed in acetone for 10 minutes, air dried at room temperature for 10 minutes, and blocked with 10% donkey serum at room temperature for 20 minutes. For ICAM-1, fibronectin, and ED-1 staining, the primary polyclonal antibody to ICAM-1 (R&D systems), the extracellular domain of fibronectin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or ED-1 (Chemicon International, Inc., Billerica, MA, USA), was diluted 1:100 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, a secondary

donkey anti-goat antibody was added for 20 minutes and the slides were then washed and incubated with a tertiary PAP complex for 20 minutes. DAB was added for 2 minutes and the slides were counterstained with hematoxylin. A semi-quantitative score for measuring the intensity of ICAM-1 and fibronectin staining within the glomeruli and tubulointerstitial area was determined by examining at least 20 glomeruli under X 400 magnification and 20 tubulointerstitial fields under X 200 magnification, respectively, and by a digital image analyzer (MetaMorph version 4.6r5, Universal Imaging Corp., Downingtown, PA, USA) as previously described[24].

The degree of staining was semi-quantitated on a scale of 0-4+. The staining score was obtained by multiplying the intensity of staining by the percentage of glomeruli or tubulointerstitium staining for that intensity and these numbers were then added for each experimental animal to give the staining score [Σ=(Intensity of staining) X (% of glomeruli or tubulointerstitium with that intensity)]. The number of ED-1 positive cells was counted in at least 20 glomeruli and 20 fields of the tubulointerstitium/section under X 400 magnification.

Similarly, the degree of glomerular and tubulointerstitial fibrosis was assessed by examining at least 20 glomeruli and 20 tubulointerstitial fields of Masson's trichrome-stained renal tissues and the mean percentages of the collagen-positive areas were obtained from each rat.

9. Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analyzed using the Kruskal-Wallis non-parametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

III. RESULTS

1. Animal studies

A. Animal data

All animals gained weight over the 3-month experimental period, but body weight was highest in C rats (593±11 g). The ratio of kidney weight to body weight in DM rats (1.17±0.15%) was significantly higher than those in C (0.58±0.05%), C+GLA (0.53±0.06%) ($p<0.01$), and DM+GLA rats (0.88±0.11) ($p<0.05$). The mean blood glucose levels of C, C+GLA, DM, and DM+GLA rats were 104.1±3.9, 97.6±3.5, 489.5±14.0, and 474.0±13.0 mg/dl, respectively ($p<0.01$). Compared to the C group (0.35±0.07 mg/day), 24-hour urinary albumin excretion at 3 months was significantly higher in DM rats (2.51±0.28 mg/day) ($p<0.01$), and GLA treatment significantly reduced albuminuria in DM rats (1.11±0.12 mg/day) ($p<0.05$) (Table 1).

Table 1. Animal data of the four groups

	C (N=8)	C+GLA (N=8)	DM (N=8)	DM+GLA (N=8)
Body Wt (g)	593±11	584±14	328± 9*	335±8*
Kidney Wt/Body Wt (%)	0.58±0.05	0.53±0.06	1.17±0.15*	0.88±0.11#
Blood glucose (mg/dl)	104.1± 3.9	97.6± 3.5	489.5±14.0*	474.0±13.0*
24-hour UAE (mg/day)	0.35±0.07	0.31±0.09	2.51±0.28*	1.11±0.12#

*, $p<0.01$ vs. C and C+GLA group; #, $p<0.05$ vs. DM group.

Wt: weight; UAE: urinary albumin excretion

B. Effect of GLA on renal cortical MCP-1 mRNA and protein expression

Renal MCP-1 mRNA expression assessed by real-time PCR was significantly increased in DM compared to C rats ($p<0.01$), and this increase in MCP-1 mRNA expression was significantly inhibited by the administration of GLA ($p<0.05$). The MCP-1 mRNA/18s rRNA ratio was 2.1-fold higher in DM compared to C kidney, and GLA treatment significantly abrogated this increase by 65.7% (Figure 1). The levels of renal MCP-1 assessed by ELISA were also significantly higher in DM relative to C rats (563.5±42.9 vs. 287.1±22.3 ng/μg, $p<0.01$), and the increase in MCP-1 levels in DM rats was significantly ameliorated by GLA treatment (354.9±31.3 ng/μg, $p<0.05$) (Figure 2).

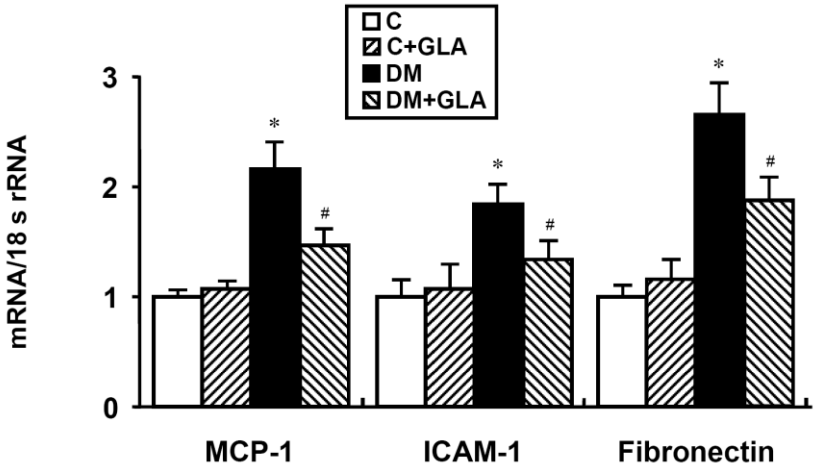

Figure 1. Renal MCP-1, ICAM-1, and fibronectin mRNA/18s rRNA ratios in C, C+GLA, DM, and DM+GLA rats. There were 2.1-fold increase in MCP-1 mRNA/18s rRNA, 1.8-fold increase in ICAM-1 mRNA/18s rRNA, and 2.7-fold increase in fibronectin mRNA/18s rRNA ratios in DM compared to C rats, and GLA treatment significantly abrogated these increases in mRNA/18s rRNA ratios in DM rats.

*; $p<0.01$ vs. C and C+GLA groups, #; $p<0.05$ vs. DM group

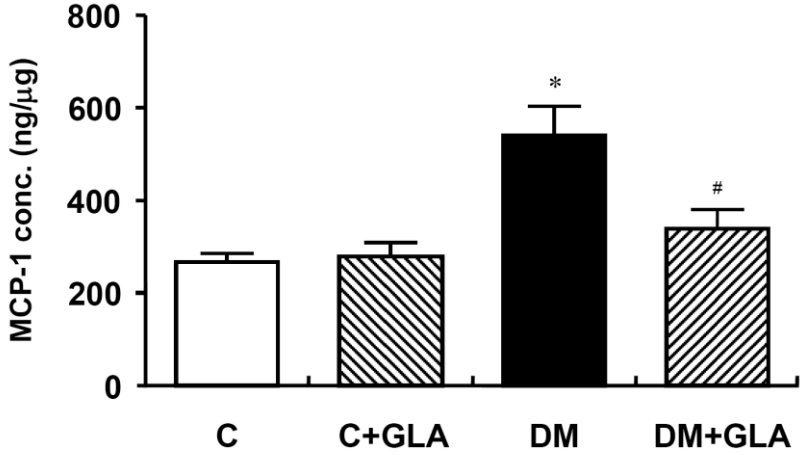

Figure 2. Renal MCP-1 protein levels in C, C+GLA, DM, and DM+GLA rats. There was 2.0-fold increase in renal MCP-1 protein levels in DM compared to C rats, and this increase in DM rats was significantly ameliorated by GLA treatment.

*; $p<0.01$ vs. C and C+GLA groups, #; $p<0.05$ vs. DM group

C. Effect of GLA on renal cortical ICAM-1 mRNA and protein expression

As seen in Figure 1, the ratio of ICAM-1 mRNA/18s rRNA was significantly higher in DM compared to C and C+GLA kidney ($p<0.01$), and GLA treatment significantly attenuated this increase in renal ICAM-1 mRNA expression in DM rats ($p<0.05$). Similarly, renal ICAM-1 protein expression assessed by Western blot was also significantly increased in DM relative to C and C+GLA rats ($p<0.01$), and this increase was inhibited by 52.9% with GLA treatment ($p<0.05$) (Figure 3). In addition, immunohistochemical staining for ICAM-1 confirmed the real-time PCR and Western blot findings. There was a significant increase in glomerular and tubulointerstitial ICAM-1 staining in the DM compared to the C and C+GLA groups, and the administration of GLA significantly abrogated this increase in ICAM-1 protein expression in DM rats (Figure 4, 5).

D. Effect of GLA on renal cortical fibronectin mRNA and protein expression

To elucidate the effect of GLA on ECM accumulation in experimental diabetic nephropathy, fibronectin mRNA and protein expression were determined with the renal cortical tissue. Renal fibronectin mRNA/18s rRNA ratio was 2.7-fold higher in DM compared to C rats ($p<0.01$), and this increase was ameliorated by 76.2% with GLA treatment ($p<0.01$) (Figure 1). Fibronectin protein expression showed a similar pattern to the mRNA expression (Figure 3). Immunohistochemical staining also revealed that fibronectin protein expression within glomeruli and tubulointerstitium was significantly increased in DM relative to C rats, and GLA treatment significantly attenuated glomerular and tubulointerstitial fibronectin accumulation in DM rats (Figure 4, 5).

E. Effect of GLA on macrophage accumulation

The number of macrophages within glomeruli and tubulointerstitium assessed by immunohistochemical staining with ED-1 antibody was significantly higher in DM compared to C rats (27.8±4.3 vs. 3.6±0.8, $p<0.005$), and GLA treatment significantly abrogated the number of ED-1-positive cells in DM rats (10.1±1.2) ($p<0.01$) (Figure 5).

F. Effect of GLA on glomerular and tubulointerstitial fibrosis

Glomerular and tubulointerstitial fibrosis assessed by Masson's trichrome staining were significantly severer in DM compared to C rats (glomerular area; 11.4±2.7 vs. 2.6±0.3, $p<0.01$, tubulointerstitial area; 23.6±4.1 vs. 3.6±0.5, $p<0.005$), and GLA treatment significantly ameliorated the extent of glomerular and tubulointerstitial fibrosis in DM rats (5.7±1.1 and 8.8±1.1), respectively ($p<0.01$) (Figure 6).

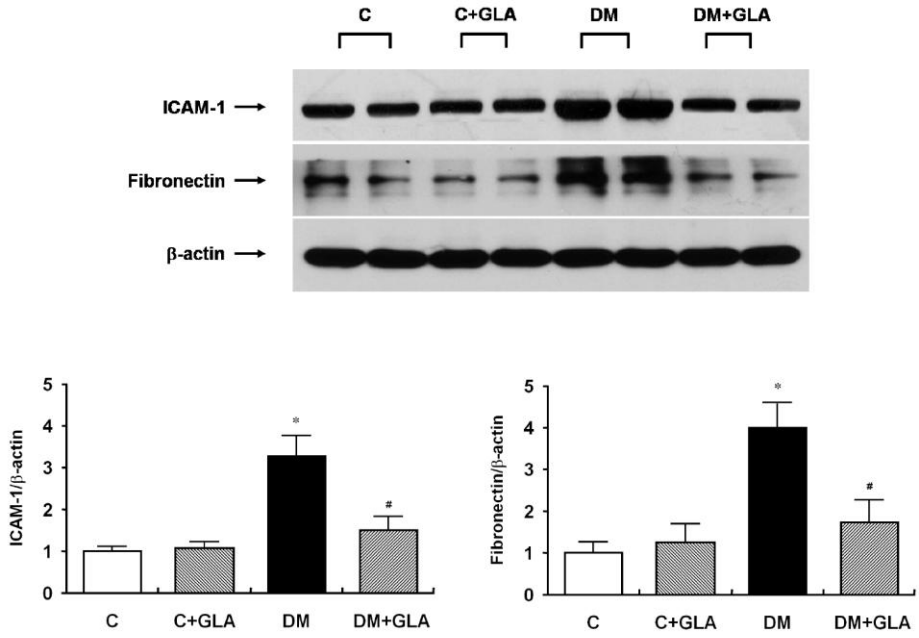

Figure 3. Renal ICAM-1 and fibronectin protein expression in C, C+GLA, DM, and DM+GLA rats. There were 3.2-fold increase in ICAM-1 and 3.7-fold increase in fibronectin protein expression in DM compared to C rats, and these increases were significantly attenuated by the administration of GLA.

*; $p<0.01$ vs. C and C+GLA groups, #; $p<0.05$ vs. DM group

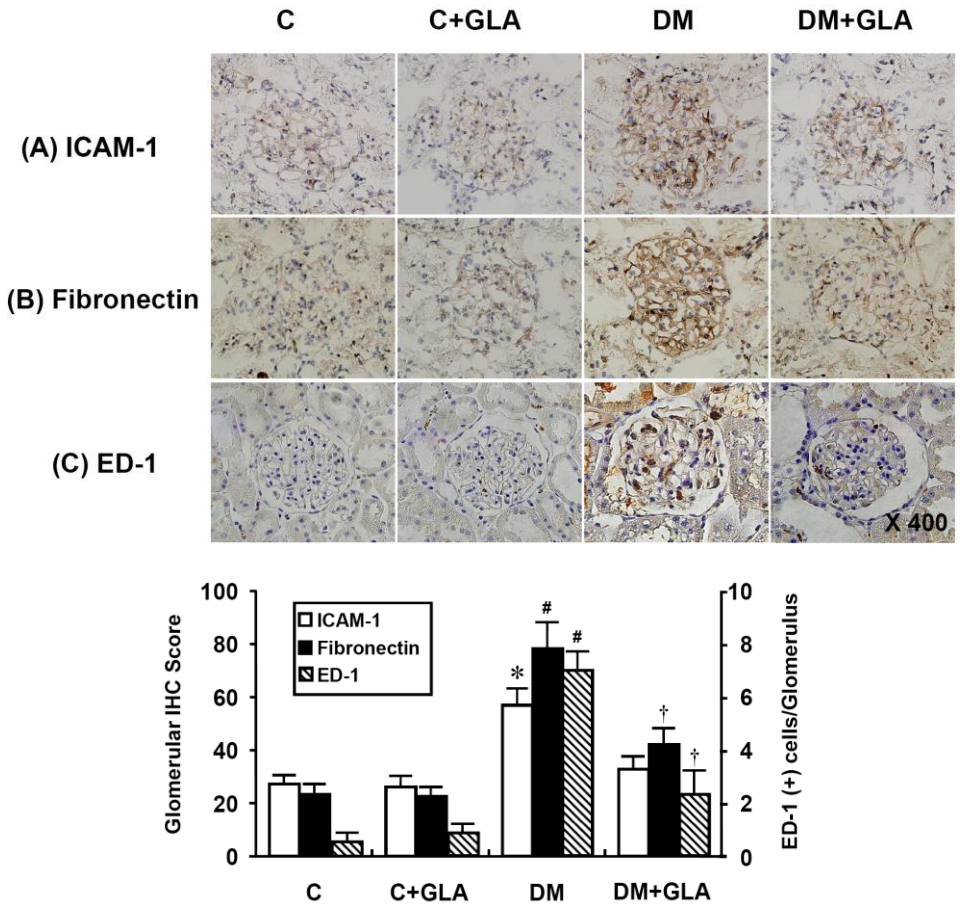

Figure 4. Immunohistochemical staining for glomerular ICAM-1, fibronectin, and ED-1 (as a marker of macrophage) in C, C+GLA, DM, and DM+GLA rats. Glomerular ICAM-1 (A) and fibronectin (B) stainings were significantly increased in DM compared to C rats, and GLA treatment significantly inhibited these increases in DM rats. The number of ED-1-positive cells (C) was significantly higher in DM compared to C rats, and GLA treatment significantly abrogated the number of glomerular macrophages in DM rats. (X 400).

*; $p<0.05$ vs. other groups, #; $p<0.01$ vs. C and C+GLA groups, †; $p<0.05$ vs. DM group

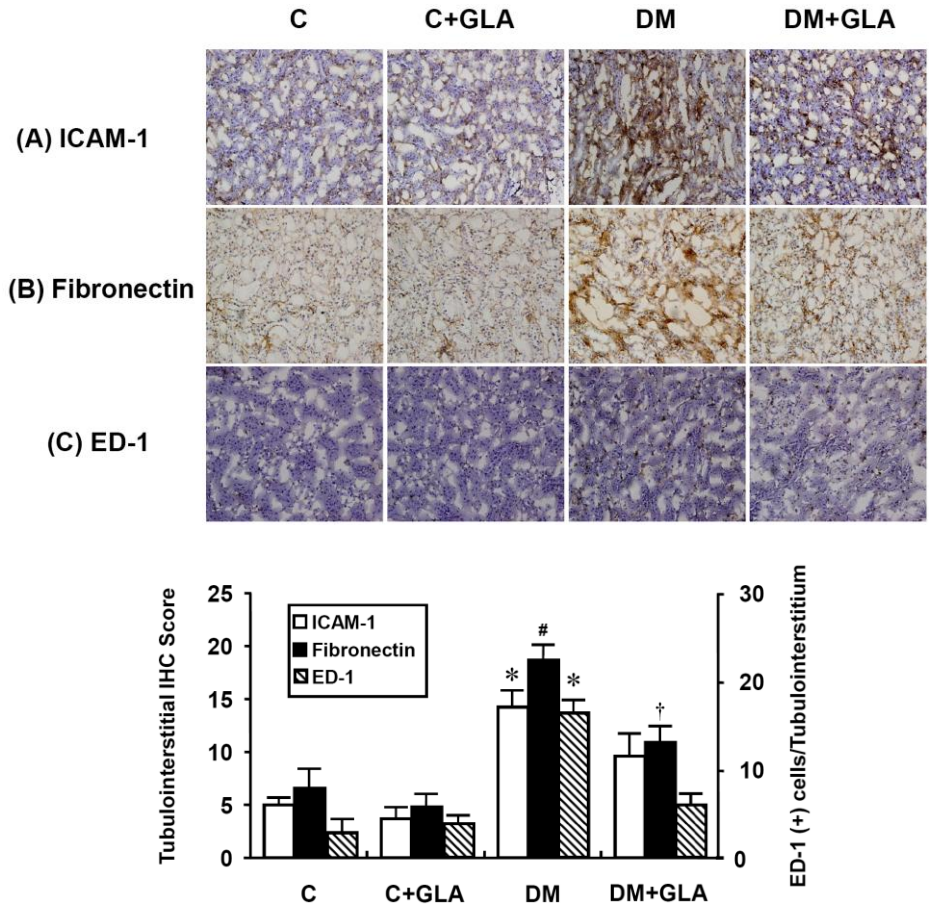

Figure 5. Immunohistochemical staining for tubulointerstitial ICAM-1, fibronectin, and ED-1 in C, C+GLA, DM, and DM+GLA rats. Tubulointerstitial ICAM-1 (A) and fibronectin (B) stainings were significantly increased in DM compared to C rats, and GLA treatment significantly ameliorated these increases in DM rats. The number of ED-1-positive cells (C) was significantly higher in DM compared to C rats, and GLA treatment significantly attenuated the number of tubulointerstitial macrophages in DM rats. (X 400).

*; $p<0.05$ vs. other groups, #; $p<0.01$ vs. C and C+GLA groups, †; $p<0.05$ vs. DM group

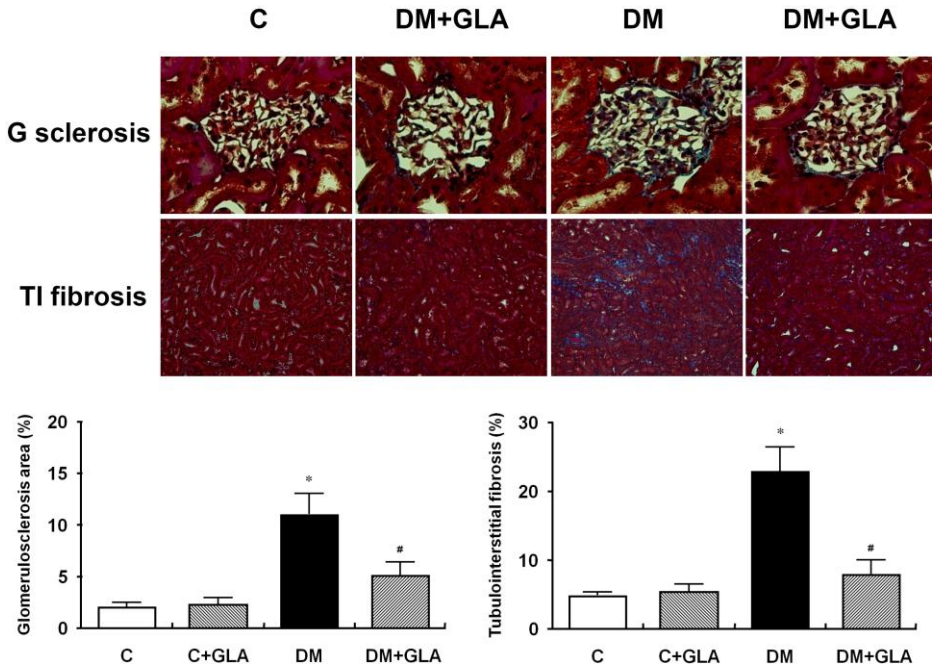

Figure 6. Glomerular and tubulointerstitial fibrosis assessed by Masson's trichrome staining in C, C+GLA, DM, and DM+GLA rats. Glomerular and tubulointerstitial fibrosis were significantly severer in DM compared to C rats, and GLA treatment significantly abrogated these changes in DM rats.

*; $p<0.01$ vs. C and C+GLA groups, #; $p<0.05$ vs. DM group

2. Cell culture studies

A. Effect of GLA on MCP-1 mRNA and protein expression

MCP-1 mRNA expression assessed by real-time PCR was significantly increased in HG-stimulated mesangial cells and NRK-52E cells ($p<0.01$), and this increase in MCP-1 mRNA expression was significantly abrogated by GLA treatment ($p<0.05$). Compared to NG cells, the MCP-1 mRNA/18s rRNA ratios were 2.0- and 2.1-fold higher in mesangial cells and NRK-52E cells exposed to HG medium ($p<0.01$), respectively, and GLA treatment significantly ameliorated these increases in a dose-dependent manner ($p<0.05$) (Figure 7). The levels of MCP-1 protein in conditioned culture media assessed by ELISA showed a similar pattern to the mRNA expression (Figure 8).

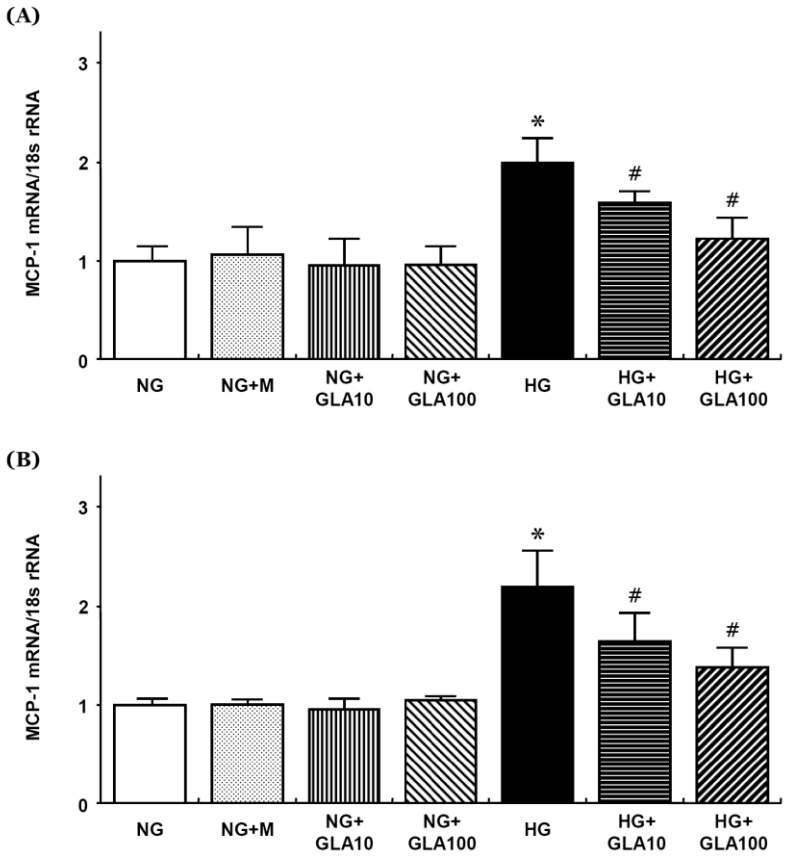

Figure 7. MCP-1 mRNA/18s rRNA ratios in mesangial cells (A) and NRK-52E cells (B) exposed to 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), NG+10 or 100 μM GLA (NG+GLA), 30 mM glucose (HG), and HG+10 or 100 μM GLA (HG+GLA). There were 2.0- and 2.1-fold increases in MCP-1 mRNA/18s rRNA ratios in HG-stimulated mesangial cells and NRK-52E cells, respectively, compared to NG cells, and these increases in MCP-1 mRNA expression were significantly ameliorated by GLA treatment in a dose-dependent manner.

*; $p<0.01$ vs. NG, NG+M, and NG+GLA groups, #; $p<0.05$ vs. HG group

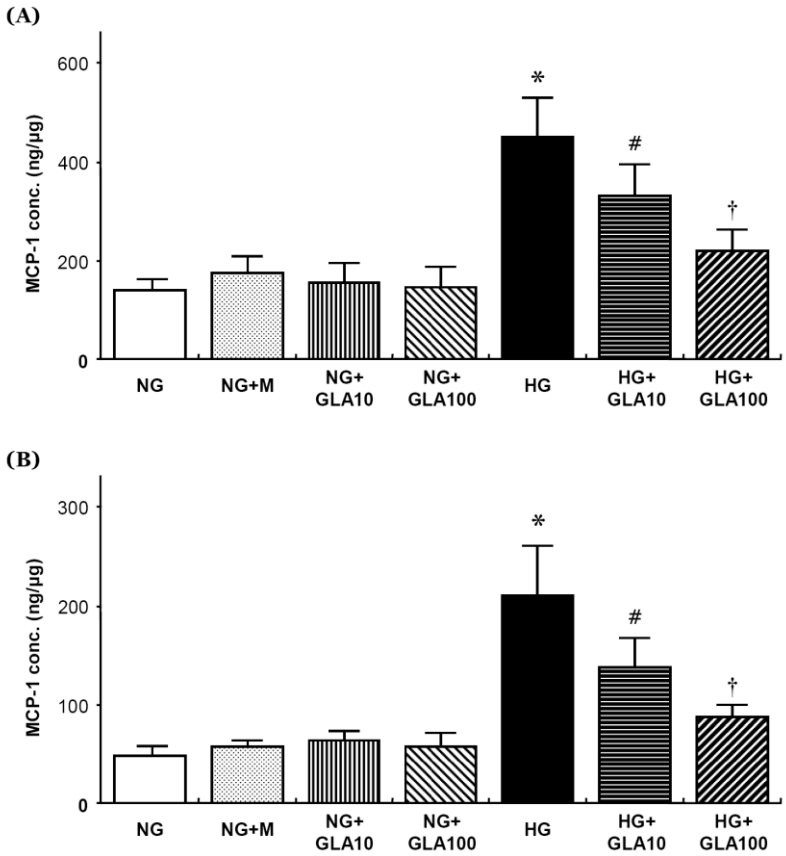

Figure 8. MCP-1 levels in conditioned culture media from mesangial cells (A) and NRK-52E cells (B) exposed to 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), NG+10 or 100 μM GLA (NG+GLA), 30 mM glucose (HG), and HG+10 or 100 μM GLA (HG+GLA). There were 2.4- and 3.6-fold increases in MCP-1 levels in HG-stimulated mesangial cells and NRK-52E cells, respectively, compared to NG cells, and GLA significantly attenuated these increases in MCP-1 levels in a dose-dependent manner.

*; $p<0.01$ vs. NG, NG+M, and NG+GLA groups, #; $p<0.05$ vs. HG group, †; $p<0.01$ vs. HG group

B. Effect of GLA on ICAM-1 mRNA and protein expression

High glucose significantly induced ICAM-1 mRNA and protein expression in mesangial cells and NRK-52E cells. Compared to NG cells, the ICAM-1 mRNA/18s rRNA ratios were 2.2- and 1.8-fold higher in HG-stimulated mesangial cells and tubular epithelial cells ($p<0.01$), respectively, and these increases were significantly attenuated by 60.9% and 62.3%, respectively, with 10 μM GLA treatment, and by 70.7% and 76.1%, respectively, with 100 μM GLA treatment (Figure 9).

GLA also significantly inhibited HG-induced ICAM-1 protein expression in cultured mesangial cells and NRK-52E cells (Figure 10).

C. Effect of GLA on fibronectin mRNA and protein expression

Fibronectin mRNA/18s rRNA ratios were significantly increased in HG-stimulated mesangial cells and NRK-52E cells relative to NG cells by 154.4% and 122.2% ($p<0.01$), respectively, and these increases were significantly abrogated by GLA in a dose-dependent manner ($p<0.05$) (Figure 11). Fibronectin protein expression showed a similar pattern to the mRNA expression (Figure 10).

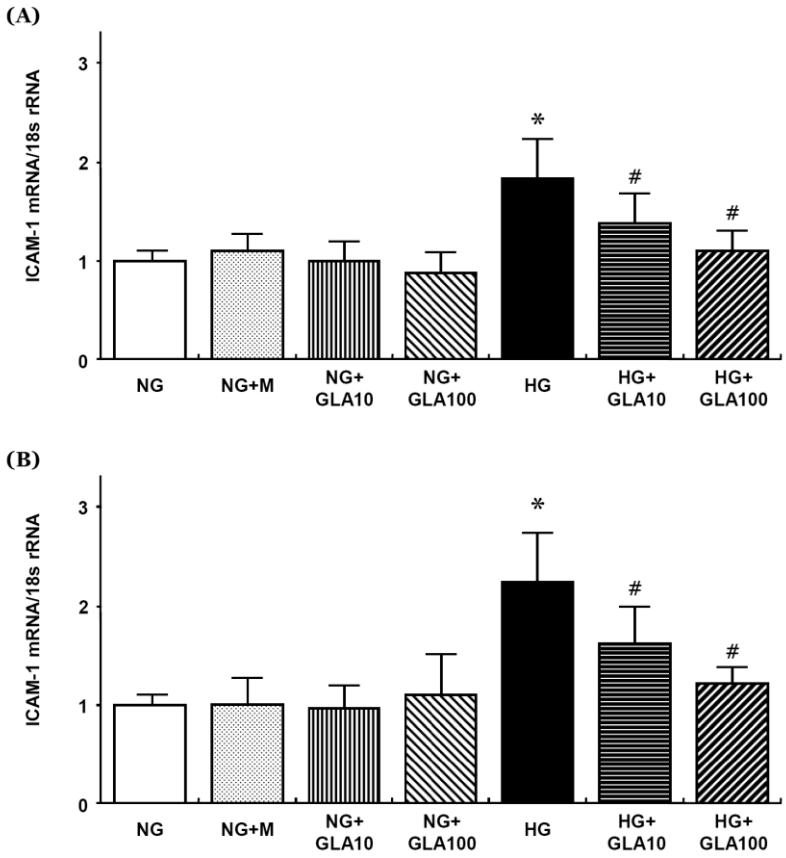

Figure 9. ICAM-1 mRNA/18s rRNA ratios in mesangial cells (A) and NRK-52E cells (B) exposed to 5.6 mM glucose (NG), NG+24.4 mM mannitol (NG+M), NG+10 or 100 μM GLA (NG+GLA), 30 mM glucose (HG), and H+10 or 100 μM GLA (HG+GLA). There were 2.2- and 1.8-fold increases in ICAM-1 mRNA/18s rRNA ratios in HG-stimulated mesangial cells and NRK-52E cells, respectively, compared to NG cells, and these increases in ICAM-1 mRNA/18s rRNA ratios were significantly abrogated by the administration of GLA.

*; $p<0.01$ vs. NG, NG+M, and NG+GLA groups, #; $p<0.05$ vs. HG group

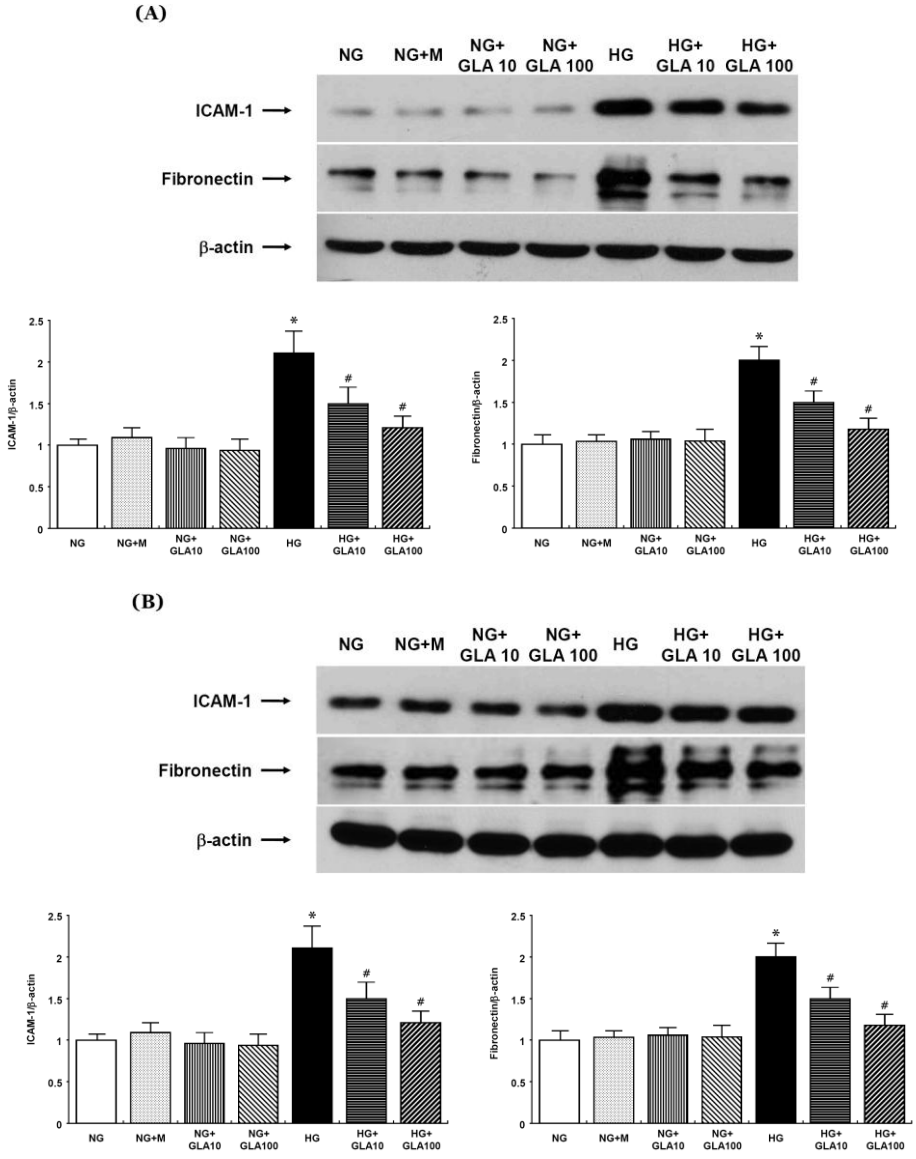

Figure 10. A representative Western blots of ICAM-1 and fibronectin in cultured mesangial cells (A) and NRK-52E cells (B) (N=5). There were significant increases in ICAM-1 and fibronectin protein expression in HG-stimulated cells compared to NG cells, and these increases were significantly ameliorated with GLA treatment.

*; $p<0.01$ vs. NG, NG+M, and NG+GLA groups, #; $p<0.05$ vs. HG group, †; $p<0.01$ vs. HG group

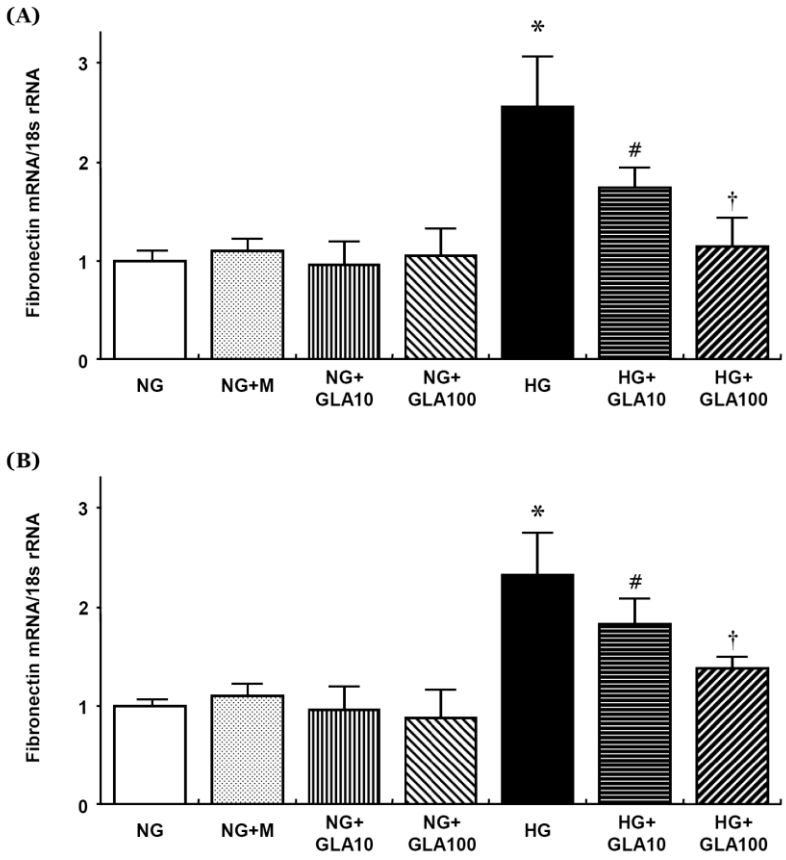

Figure 11. Fibronectin mRNA/18s rRNA ratios in mesangial cells (A) and NRK-52E cells (B) exposed to 5.6 mM glucose(NG), NG+24.4 mM mannitol (NG+M), NG+10 or 100 μM GLA (NG+GLA), 30 mM glucose (HG), and HG+10 or 100 μM GLA (HG+GLA). There were 2.5-fold and 2.2-fold increases in fibronectin mRNA/18s rRNA ratios in HG-stimulated mesangial cells and NRK-52E cells, respectively, compared to NG cells, and GLA treatment significantly attenuated these increases in fibronectin mRNA/18s rRNA ratios in a dose-dependent manner.

*; $p<0.01$ vs. NG, NG+M, and NG+GLA groups, #; $p<0.05$ vs. HG group, †; $p<0.01$ vs. HG group

IV. DISCUSSION

In the present study, I demonstrate that GLA has a renoprotective effect via its anti-inflammatory and anti-fibrotic actions in experimental diabetic nephropathy. In addition, the results of this study suggest that the anti-inflammatory effect of GLA under diabetic conditions is partly mediated by inhibiting the increases in MCP-1 and ICAM-1 expression under diabetic conditions.

Even though the diabetic milieu *per se*, hemodynamic changes, and local growth factors such as AII and TGF-β are considered mediators in the pathogenesis of diabetic nephropathy[11,25-27], recent studies suggest that an inflammatory mechanism may also contribute to the development of diabetic nephropathy based on the pathological findings of inflammatory cell infiltration in diabetic kidney[10,23-25]. Monocytes/macrophages are the major inflammatory cells found in diabetic kidney[28]. They are extravasculated from the bloodstream and attracted to the target tissue through a process mediated by various chemokines and adhesion molecules such as MCP-1 and ICAM-1[29,30]. In the kidney, MCP-1 is expressed in mesangial cells and tubular epithelial cells and is known to be involved in the pathogenesis of various renal diseases, including diabetic nephropathy[30]. Previous studies have demonstrated that plasma MCP-1 levels are increased in type 1 diabetes with microalbuminuria[31] and that urinary levels of MCP-1 are increased in accordance with the extent of albuminuria[10,32]. Renal expression of ICAM-1, a cell surface glycoprotein that plays a major role in the regulation of interactions with immune cells and whose expression is upregulated at the sites of inflammation, is also

known to be increased in experimental type 1 and type 2 diabetic animals. These findings suggest that MCP-1 and ICAM-1 may play an important role in the pathogenesis of diabetic nephropathy via inducing inflammatory cell infiltration[11,33]. Once recruited monocytes/macrophages are activated, they release lysosomal enzymes, nitric oxide, reactive oxygen species, platelet-derived growth factor (PDGF), tumor necrosis factor-α, interleukin (IL)-1, and TGF-β, and in turn promote renal injury[8,34,35]. PDGF stimulates fibroblast proliferation[36,37] and IL-1 induces the expression of TGF-β, the most well-known profibrotic cytokine, in fibroblasts[38]. In experimental diabetic nephropathy, various anti-inflammatory agents inhibited not only inflammatory cell infiltration via abrogating the increases in MCP-1 and ICAM-1 expression but also ameliorated ECM accumulation[28]. In addition, renal fibrosis was significantly inhibited along with less inflammatory cell infiltration in MCP-1- and ICAM-1-deficient diabetic mice[39]. Taken together, it is suggested that the inhibition of inflammatory cell recruitment may lead to an attenuation of ECM accumulation. In this study, I demonstrate that MCP-1 and ICAM-1 expression were increased in experimental diabetic nephropathy, which were associated with glomerular and tubulointerstitial fibrosis, and in high glucose-stimulated mesangial cells and tubular epithelial cells, and these increases under diabetic conditions were inhibited by GLA treatment. Taken together, the anti-inflammatory and anti-fibrotic effects of GLA in diabetic nephropathy may be partly attributed to the suppression of MCP-1 and ICAM-1 expression by GLA, by

which inflammatory cell infiltration is abrogated, and in turn ECM accumulation may be ameliorated.

PUFAs are important constituents of all cell membranes. Since PUFAs are not synthesized in humans, they can be obtained only by diet[40]. There are two classes of PUFAs; ω-3 and ω-6, designated according to their carbon ring structure[17,19,41], and accumulating evidence has shown that these PUFAs are beneficial to health and a number of various diseases[42]. In cases of kidney disease, PUFAs have been reported to exert beneficial effects on IgA nephropathy[43], chronic renal failure, and diabetic nephropathy via anti-oxidant, anti-inflammation, and anti-fibrotic mechanisms[44]. In contrast, the effect of GLA, a member of the ω-6 PUFA family, on kidney diseases has been less explored. Ingram et al observed that administration of borage oil (BO), which is a rich source of GLA, was effective in the rat 5/6-renal-ablation model[21]. BO prevented the increases in blood pressure and proteinuria, the rise in plasma cholesterol levels, and the decline in glomerular filtration rates. In addition, glomerular macrophage infiltration, mesangial expansion, and glomerulosclerosis were attenuated in BO-treated rats compared to the control diet group. Meanwhile, since these glomerular changes are also characteristic in diabetic nephropathy, activities of δ-5 and δ-6 desaturase are decreased along with low levels of GLA and DGLA in diabetes[45,46], and GLA has been useful in diabetic neuropathy[22,23,47], supplementation of GLA and/or DGLA may also be of benefit in diabetic nephropathy. However, the effect of ω-6 PUFA has never been elucidated in diabetic nephropathy. The results of the present study show for the first time that

GLA inhibits inflammatory cell infiltration and ECM accumulation in experimental diabetic kidney, suggesting the usefulness of GLA in patients with diabetic nephropathy.

Even though the underlying mechanisms of the anti-inflammatory effect of GLA in this study are not completely understood, several plausible explanations can be implicated. First, PUFAs, including GLA, are known to serve as endogenous ligands of peroxisome proliferator activated receptors (PPARs) and to bind and activate all PPARs isoforms[48,49]. Additionally, 15-HETE, one of the metabolites of GLA, are reported to upregulate nuclear PPAR-γ expression[50]. PPARs is a regulator of lipid metabolism[51] and is closely associated with insulin action[52]. Moreover, PPARs participate in the regulation of inflammatory response by inhibiting monocyte expression of proinflammatory cytokines such as interleukin-6 (IL-6), IL-1β, and TNF- α[53,54]. Furthermore, PPAR-γ attenuates the nuclear factor-κB-mediated transcriptional activation of proinflammatory genes[55]. Recent studies have also demonstrated that PPAR-γ agonist exerts the renoprotective effect through anti-inflammatory mechanism in diabetic nephropathy[56,57]. Taken together, GLA as a ligand of PPARs and its metabolite as a upregulator of PPAR-γ could contribute to the anti-inflammatory effect of GLA in diabetic nephropathy. Second, a small amount of DGLA can be converted to prostacylin (PGI2) and prostaglandin E1 (PGE1) by δ-5 desaturases. Since PGI2 and PGE1 inhibits platelet aggregation, and PGI2 analogue abrogates glomerular hyperfiltration and macrophage infiltration in the diabetic kidney[58], the effect of GLA may be in part attributed to these

consequences. Third, 15-HETE markedly inhibits the generation of leukotriene, which is a potent pro-inflammatory mediator via stimulating adhesion molecule expression and macrophage infiltration[59]. Collectively, the anti-inflammatory effect of GLA seems to be attributed to modulating the biological cascade at multiple sites by itself and/or its metabolites. Prolonged use of anti-inflammatory drugs may be harmful and is not appropriate for chronic use due to many systemic side effects in patients with chronic metabolic disturbances such as diabetic nephropathy. However, since the activities of δ-5 and δ-6 desaturase are already reduced in diabetes and toxic effects of GLA as medicinal oil have not been reported, chronic administration of GLA, even at a high dose, may not induce the accumulation of its metabolites and thus will be not harmful.

In summary, the results of my study demonstrate that GLA exerts anti-inflammatory and anti-fibrotic effects in experimental diabetic nephropathy and in high glucose-stimulated renal cells, suggesting that GLA supplementation could be a valuable therapeutic option for the treatment of diabetic nephropathy.

V. CONCLUSION

In this study, I investigated the effect of gamma linolenic acid (GLA) on inflammation and ECM accumulation in experimental diabetic nephropathy and in HG-stimulated renal cells.

1. Twenty-four-hour urinary albumin excretion at 3 months was significantly higher in DM compared to C rats, and GLA treatment significantly reduced albuminuria in DM rats.

2. MCP-1 mRNA expression was significantly increased in DM kidney and in HG-stimulated mesangial cells and tubular epithelial cells compared to C kidney and NG cells, respectively, and this increase was significantly abrogated by GLA treatment. The levels of MCP-1 in the renal cortical tissue and conditioned culture media showed a similar pattern to the mRNA expression.

3. ICAM-1 mRNA and protein expression was significantly increased in DM kidney and in HG-stimulated mesangial cells and tubular epithelial cells compared to C kidney and NG cells, respectively, and these changes were significantly ameliorated by GLA treatment.

4. Fibronectin mRNA and protein expression were significantly increased in DM kidney and in HG-stimulated mesangial cells and tubular epithelial cells compared to C kidney and NG cells, respectively, and GLA treatment significantly attenuated these increases in fibronectin expression.

5. Immunohistochemical staining revealed that the number of ED-1 (+) cells within glomeruli and tubulointerstitium was significantly higher in DM compared

to C rats, and this increase in ED-1 (+) cells was significantly abrogated by the administration of GLA.

6. The extent of glomerular and tubulointerstitial fibrosis assessed by Mason's trichrome staining was significantly severer in DM compared to C kidney, and this change was significantly ameliorated by GLA treatment.

In conclusion, GLA attenuates not only inflammation via inhibiting enhanced MCP-1 and ICAM-1 expression but also ECM accumulation in diabetic nephropathy.

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ABSTRACT (IN KOREAN)

실험적 당뇨병성 신병증에서 gamma linolenic acid 가 염증반응 및 세포외 기질 축적에 미치는 영향

<지도교수 강신욱>

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배 경 : 당뇨병성 신병증은 투석이나 이식이 필요한 말기 신부전증의 가장 많은 원인 질환이다. 당뇨병성 신증에서 특징적인 병리학적 변화는 사구체 및 세뇨관의 비후와 세포외 기질의 축적이며, 이러한 변화와 발생 및 진행에는 고혈당에 의해 유도된 각종 성장 인자들의 활성화, 세포 외 기질의 생성 증가, 세포 외 기질 단백 분해 효소의 생성 감소 등이 관여하는 것으로 알려져 있다. 최근에는 당뇨병 환자나 실험적 당뇨 동물 모델의 사구체 및 세뇨관-간질 내에 염증세포의 침윤이 관찰될 뿐만

아니라 염증 반응과 밀접한 관련이 있는 monocyte chemotactic protein-1 (MCP-1)과 intracellular adhesion molecule-1 (ICAM-1)의 발현이 당뇨 신장 내에 증가되어 있다는 연구 결과들이 발표되면서 염증반응이 당뇨병성 신병증의 병태생리에 중요한 역할을 할 것으로 생각되어지고 있다.

Gamma linolenic acid (GLA)는 필수 지방산으로, 식물성 oil 성분으로 보충하거나 불포화 지방산인 linoleic acid (LA)에서 δ-5 및 δ-6 desaturase를 통해 형성되는 것으로 알려져 있다. 당뇨병이나 알코올 섭취 등은 이들 효소의 활성을 억제시킴으로써 상대적으로 GLA의 감소가 유발되며, 이러한 GLA의 감소가 당뇨병성 신경병증 및 심혈관 합병증과 관련이 있다는 보고가 있다. GLA는 고포도당으로 자극한 신경세포에서 advanced glycation endproduct의 생성을 감소시켜 당뇨병성 신경병증을 개선시키며, 아토피성 피부염 등의 피부 질환에서는 염증반응을 호전시키는 것으로 알려져 있다. 또한, GLA 및 LA는 여러 세포에서 다양한 자극에 의해 유도되는 염증반응을 개선시키는 것으로 보고되고 있으며, 이러한 과정에 특히 Mitogen Activated Protein Kinase나 Peroxisome proliferator-activated receptor-gamma pathway가 관여하는 것으로 알려져 있다.

신장 질환의 경우, GLA가 신섬유화 동물 모델에서 대식세포의 침윤과 메산지움의 확장을 억제시켰을 뿐만 아니라 신기능의 악화를 예방하였다는 보고는 있었으나, 당뇨병성 신병증에서 GLA의 효과에

대한 연구는 전무한 실정이다. 이에 본 연구자는 당뇨병성 신병증 동물 모델과 고포도당으로 자극한 신세포를 대상으로 GLA가 염증반응과 밀접한 관련이 있는 MCP-1과 ICAM-1의 발현에 미치는 영향과 대표적인 세포외 기질인 fibronectin의 발현에 미치는 영향을 연구하였다.

방 법 : 생체 내 실험으로는 Sprague-Dawley 백서 32마리를 대조군 (8마리), 당뇨군 (8마리), 그리고 GLA (450 mg/kg/day, 구강 투여), 투여 대조군 (8마리) 및 당뇨군 (8마리)으로 나누어 사육한 뒤, 당뇨 유발 12주 후에 신장 피질 조직을 분리하였다. 또한 생체 외 실험으로는 백서 메산지움 세포와 세뇨관 상피세포를 정상 포도당군 (5.6 mM), 정상 포도당 + 만니톨군 (24.4 mM), 정상 포도당+ GLA (10 or 100 μM) 처치군, 고포도당 (30 mM), 그리고 고포도당+ GLA (10 or 100 μM) 처치군으로 나누어 24시간 배양하였다. 신장 조직 및 배양액 내 monocyte chemotactic protein-1 (MCP-1) 농도는 ELISA를 이용하여 측정하였으며, 신장 조직 및 배양세포 내 MCP-1 mRNA 발현은 real-time PCR을 이용하여 분석하였다. ICAM-1과 fibronectin의 mRNA와 단백 발현도 각각 real-time PCR과 Western blot으로 확인하였다. 또한, 신장 조직 내 ICAM-1과 fibronectin의 단백 발현, 그리고 신장 내 침윤 대식세포는 면역조직화학염색법으로 관찰하였고, 사구체 및 세뇨관-간질 내 섬유화 정도는 Masson's trichrome 염색 (MT염색)으로 관찰하였다.

결 과 :

1. 24시간 뇨알부민 배설은 대조군에 비하여 당뇨군에서 유의하게 높았으며 ($p<0.05$), GLA 투여 당뇨군에서는 당뇨군에 비하여 뇨알부민 배설 증가가 의미있게 억제되었다.
2. 신장 피질 조직 및 메산지움 세포와 세뇨관 상피세포 내에서 MCP-1의 mRNA발현 및 단백은 당뇨군과 고포도당군에서 각각 대조군과 정상 포도당군에 비하여 의의있게 증가되었으며, GLA 투여로 MCP-1 mRNA 및 단백의 발현 증가가 유의하게 억제되었다.
3. ICAM-1 mRNA와 단백 발현도 당뇨 신장 조직 및 고포도당으로 자극한 메산지움 세포와 세뇨관 상피세포에서 각각 대조군과 정상 포도당군에 비하여 의미있게 증가되었으며, GLA 처치로 이러한 ICAM-1의 발현 증가가 의의있게 억제되었다.
4. 신장 피질 조직 및 메산지움 세포와 세뇨관 상피세포 내 fibronectin의 발현은 당뇨군과 고포도당군에서 유의하게 증가되었으며, 이러한 fibronectin의 발현 증가는 GLA에 의하여 의미있게 억제되었다.
5. 면역조직화학염색상 사구체 및 세뇨관-간질 부위의 ICAM-1과 fibronectin 단백의 발현은 당뇨군에서 의의있게 증가되었으며, GLA 투여로 당뇨군에서의 발현 증가가 유의하게 억제되었다.
6. ED-1 항체를 이용한 면역조직화함염색으로 확인한 침윤

대식세포의 수는 당뇨군에서 대조군에 비하여 의미있게 많았으며, 당뇨군에서의 대식세포의 침윤은 GLA에 의하여 의의있게 감소되었다.

7. MT 염색법으로 확인한 사구체 및 세뇨관-간질 섬유화는 당뇨군에서 대조군에 비하여 유의하게 심하였으며, GLA에 의하여 의미있게 억제되었다.

결 론 : 이상의 결과로 백서 당뇨병성 신병증에서 GLA가 MCP-1과 ICAM-1의 발현 증가를 의의있게 억제시킴으로써 대식세포의 침윤이 감소되었을 것으로 생각된다. 또한, 메산지움 세포와 세뇨관 상피세포에 대한 직접적인 효과뿐만 아니라 이러한 항염증 기전을 통하여 세포외 기질의 축적이 경감되었을 것으로 사료된다. 따라서, 항염증 및 항섬유화 효과가 있는 GLA가 당뇨병성 신병증의 발생 및 진행 억제에 유용할 것으로 생각된다.

핵심 되는 말 : GLA, 당뇨병성 신병증, 메산지움 세포, 세뇨관 상피세포, 고포도당, 염증반응, MCP-1, ICAM-1, fibronectin