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Full Length Research Paper

Activation of the lectin complement pathway on human renal glomerular endothelial cells triggered by high glucose and mannose-binding lectin

Wen-Xing Fan[1], Song-Min Huang[1]*, Fang Liu[1], Gregory L. Stahl[2], Wan-Xin Tang[1], Hong-Yu Qiu[1] and Ping Fu[1]

[1]Department of Nephrology, West China Hospital, Sichuan University, Chengdu, Sichuan Province 610041, China.
[2]Center for Experimental Therapeutics & Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

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This study aimed to investigate the roles of high glucose and mannose-binding lectin (MBL) on the activation of the lectin complement pathway (LCP) on human renal glomerular endothelial cells (HRGECs) *in vitro*. Flow cytometry analysis, immunofluorescence staining and Western blot were used to detect the cell surface deposition of MBL, C3 and the membrane attack complex (MAC), as well as the code position of MBL and C3 (MBL+C3). Electrophoretic mobility shift assay (EMSA) was used to measure the activation of nuclear factor-kappaB (NF-κB). These results show that cell surface deposition of MBL, C3 and MBL+C3 after exposure to high glucose increased in both time- and dose-dependent manner ($P < 0.05$). Moreover, MBL, C3 and MBL+C3 deposition stimulated by exogenous recombinant human MBL (rhuMBL) were also observed in both time- and dose-dependent manner, peaking at 4 h and then decreasing ($P < 0.05$). Inhibition experiments indicated that the inhibitory monoclonal antibody 3F8 against human MBL (MAb 3F8) significantly abrogated the cell surface deposition of MBL, C3 and MBL+C3, attenuated MAC staining, and inhibited NF-κB activation in HRGECs ($P < 0.05$). In conclusion, high glucose and MBL played an important role on the LCP activation of HRGECs, and MAb 3F8 may represent a novel potential therapeutic strategy to block LCP activation on HRGECs.

Key words: High glucose, mannose-binding lectin, complement activation, human renal glomerular endothelial cells.

INTRODUCTION

The complement system has been activated in a cascade manner through three different pathways: the classical, the alternative and the lectin complement pathway (LCP). All three pathways merge at complement C3 and result in the formation of terminal complement complex, C5b-9 (also known as the membrane attack complex, MAC) (Collard et al., 1999). The LCP is an antibody-independent cascade initiated by binding of mannose-binding lectin (MBL) to carbohydrate structures present on the surface of bacteria, yeast, parasitic protozoa and viruses (Turner, 1996). MBL, also known as mannan-binding lectin, is a glycoprotein and belongs to the family of C-type lectins, which consists of an N-terminal cross-linking region, a collagen-like region (CLR), a neck region and a carbohydrate recognition domain (CRD). MBL is predominately synthesized by hepatocytes and secreted into the circulation, which binds to terminal sugar residues via the CRD in the serum.

*Corresponding author. Email: huang_songmin@163.com. Tel: +86-28-85422335. Fax: +86-28-85422338.

Abbreviations: MBL, Mannose-binding lectin; **LCP,** lectin complement pathway; **HRGECs,** human renal glomerular endothelial cells; **MAC,** membrane attack complex; **EMSA,** electrophoretic mobility shift assay; **NF-κB,** nuclear factor-kappaB; **rhuMBL,** recombinant human MBL.

Diabetic kidney disease (DKD) is the leading cause of end-stage kidney disease, and it is an increasing international public health problem. Data have shown that the complement system, especially the MBL, played a role in the pathogenesis of DKD (Ostergaard et al., 2005). Well-designed clinical trials in type 1 diabetic patients have indicated that both high MBL genotypes and elevated MBL serum levels were significantly more frequent in the nephropathy group than in the normoalbuminuria group (Hansen et al., 2004). Another study in type 2 diabetic patients showed that the cumulative incidence of progression to persistent micro- or macroalbuminuria was significantly higher among patients with MBL levels ≥ 1 µg/ml than that among patients with MBL levels < 1 µg/ml (Hansen et al., 2006). MBL deficient mice, such as those lacking both murine forms of MBL (MBL A and C), exhibited attenuated classic functional and physical renal changes observed in an experimental model of type 1 diabetes (Ostergaard et al., 2007). However, the molecular mechanism responsible for the potential activation of LCP in DKD is still unclear.

Numerous studies have implicated the nuclear factor-kappaB (NF-κB) activation in the development of diabetes, which was consistent with its central role in coordinating inflammatory responses (Baker et al., 2011). Moreover, evidence indicated that MBL could aggravate local and systemic inflammation through complement activation (Collard et al., 2000), and NF-κB activation could be induced by stimulation with sublytic amounts of MAC *in vitro* (Viedt et al., 2000). These findings suggest that the pathogenesis of inflammation in diabetes may be associated with LCP activation and NF-κB activation.

Our study aimed to investigate the effects of high glucose and exogenous recombinant human MBL (rhuMBL) on the LCP activation of human renal glomerular endothelial cells (HRGECs), including NF-κB activation. Inhibition experiments were performed by the addition of the inhibitory monoclonal antibody 3F8 against human MBL (MAb 3F8).

MATERIALS AND METHODS

Cells, media and reagents

This study was conducted at the Key Laboratory of Transplant Engineering and Immunology, West China Hospital, Sichuan University, Chengdu, China. All experiments were approved by the Ethics Committee of the West China Hospital of Sichuan University.

The well-characterized normal human renal glomerular endothelial cells (HRGECs) were purchased from ScienCell Research Laboratories (ScienCell, USA). HRGECs were cultured in endothelial cell medium (ECM, ScienCell) containing 5% fetal bovine serum (FBS) and 1% endothelial cell growth supplement (ECGS) at 37℃ in a 5% CO_2 atmosphere. Cells used for these experiments had undergone fewer than 6 passages, which were serially passage by brief exposure to 0.25% trypsin (Difco) and 0.04% EDTA (Sigma).

MBL-deficient human serum (MBL-Def HS) was made as previously described (Collard et al., 2000). Human serum (HS) was treated with phenylmethylsulphonyl fluoride (2mmol/L) and loaded onto a mannan-Sepharose column. The resultant MBL-Def HS was collected and dialyzed against Hanks' balanced salt solution (HBSS) containing calcium and magnesium, overnight at 4℃. Recombinant human MBL (rhuMBL) was obtained from R and D Systems, and the inhibitory MAb 3F8 antibody has been generated as previously described (Collard et al., 2000), reacting with rhuMBL with high affinity.

Study design

Effects of high glucose on the kinetic activation of LCP

Firstly, we investigated the time-effect relationship of high glucose on the cell surface deposition of MBL, C3 and MBL+C3 by flow cytometry. HRGECs were cultured in ECM containing high glucose (30 mmol/L) for different time periods (0, 12, 24, 48 or 72 h), and then cells were exposed to 30% MBL-Def HS with exogenous rhuMBL (1 µg/ml) for 4 h (Figure 1A).

Secondly, we studied the dose-effect relationship of glucose on the cell surface deposition of MBL, C3 and MBL+C3 by flow cytometry. HRGECs were cultured in ECM containing different concentrations of glucose (0, 15, 30 or 60 mmol/L) for 72 h, and then the cells were exposed to 30% MBL-Def HS with exogenous rhuMBL (1 µg/ml) for 4 h (Figure 1A).

Effects of exogenous rhuMBL on the kinetic activation of LCP

Firstly, we investigated the time-effect relationship of exogenous rhuMBL on the cell surface deposition of MBL, C3 and MBL+C3 by flow cytometry. HRGECs were cultured in ECM containing high glucose (30 mmol/L) for 72 h, and then exogenous rhuMBL (1 µg/ml) in 30% MBL-Def HS was added at different time points (0, 0.5, 1, 2, 4, 8 or 16 h) (Figure 1B).

Secondly, we studied the dose-effect relationship of exogenous rhuMBL on the cell surface deposition of MBL, C3 and MBL+C3 by flow cytometry. Cells were cultured in ECM containing high glucose (30 mmol/L) for 72 h, and then different concentrations of exogenous rhuMBL (0, 1, 2, 4 or 8 µg/ml) in 30% MBL-Def HS were added into each group for 4 h (Figure 1B).

Inhibition experiments based on MAb 3F8

HRGECs were divided into the following three groups: high-MBL group, MBL-Def group and MAb 3F8 group. Cells were cultured in ECM containing high glucose (30mmol/L) for 72 h. Then exogenous rhuMBL (8 µg/ml) in 30% MBL-Def HS was added into the high-MBL group for 4 h, 30% MBL-Def HS without exogenous rhuMBL was added into the MBL-Def group for 4 h and exogenous rhuMBL (8 µg/ml) plus MAb 3F8 (rhuMBL to MAb 3F8 molar ratio of 1:1) (Hart et al., 2005) in 30% MBL-Def HS was added into the MAb 3F8 group for 4 h (Figure 1C).

Flow cytometry assay of MBL and C3

We used flow cytometry to detect the MBL and C3 deposition on the cell surface of HRGECs. After repeated pipetting to ensure single-cell suspensions, cells (100 µl, 1×10^8 cells/ml) were stained with biotinylated mouse anti-human MBL monoclonal antibody (MBL-Biotin, D8.18, 0.4 µg/IE6 cells, Hycult) and FITC-conjugated mouse anti-human C3 monoclonal antibody (C3-FITC, 6C9, 0.2 µg/IE6 cells, Lifespan) for 1 h at room temperature. After being washed three times in phosphate-buffered saline (PBS), streptavidin-conjugated PE/Cy5 (PE/Cy5, 0.125 µg/IE6 cells, Biolegend), which was used in conjunction with biotinylated primary antibody, was

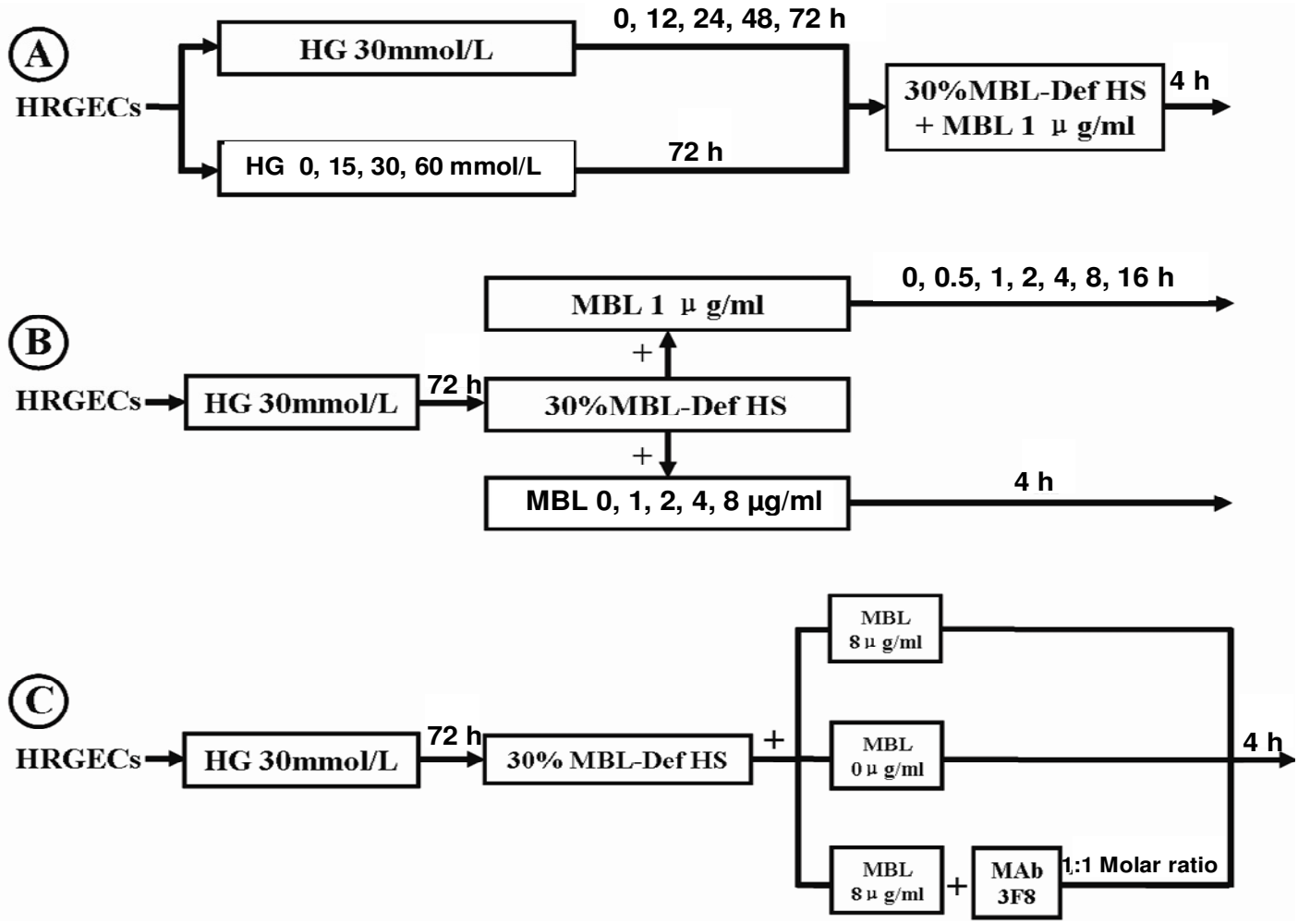

Figure 1. Schematic diagram of the whole experimental protocol *in vitro*. A: Time and dose effects of high glucose on the kinetic activation of LCP; B: Time and dose effects of exogenous rhuMBL on the kinetic activation of LCP; C: Inhibition experiments based on MAb 3F8.

added and incubated for 30 min at room temperature. The wash procedure was repeated, and the cells were resuspended to 500 µl of PBS. Cells were analyzed for coated proteins using a flow cytometer (EPICS XL, Coulter) to quantify red or green fluorescence. Positive cells were stained by MBL-Biotin and PE/Cy5 (MBL-PE/Cy5) alone, stained by C3-FITC alone or double stained by both MBL-PE/Cy5 and C3-FITC together according to experimental requirements. The fluorescence intensity of 1×10^4 cells for each sample was quantified, and the unstained cells were used as controls. The percentage of positive cells with fluorescence labeling of MBL, C3 and MBL+C3 was calculated in each group.

Immunofluorescence (IF) assay of MBL, C3 and MAC

For immunofluorescence analyses, HRGECs were grown to 70% confluency in tissue culture chamber slides. Then, the slides were washed in PBS containing calcium and magnesium, fixed in 4% paraformaldehyde for 15 min, washed again, and blocked with 10% goat serum. MBL deposition was identified using MBL-Biotin (1:100 dilution, Hycult) and PE/Cy5 (1:200 dilution, Biolegend). C3 deposition was evaluated with C3-FITC (1:200 dilution, Lifespan). MAC deposition was evaluated with mouse anti-human C5b-9 monoclonal antibody (anti-C5b-9, aE11, 1:50 dilution, Santa Cruz) and FITC-conjugated goat anti-mouse IgG (IgG-FITC, 1:100 dilution, Sigma). Cell nuclei were stained by 4', 6-diamidin-2'-phenylindol-dihydrochloride (DAPI, 1:1000 dilution, Molecular Probes). After incubation with the appropriate antibodies, the slides were washed with PBS (3 times, 10 min each) and visualized by conventional immunofluorescence microscopy (BX51, Olympus).

Western blot analysis of MBL

HRGECs (1×10^7 cells) were disrupted in 100 µl of sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated by SDS-PAGE on a 7.5% gel and were electrophoretically transferred to a nitrocellulose membrane. The membrane was treated with blocking buffer containing PBS and 3% skimmed milk, and then incubated with mouse anti-human MBL monoclonal antibody (HYB 131-01, 1:200 dilution, Abcam) overnight at 4℃. After washing four times with the same buffer containing PBS and 3% skimmed milk, the membrane was incubited with horseradish peroxidase (HRP)-conjugated goat anti-mouse polyclonal antibody (1:3000 dilution, Santa Cruz) for 2 h at room temperature. After washing again 5 times, the immunoreactivities of

antibodies were detected using an ECL system (Amersham) and bands were visualized with X-ray films (Kodak). β-actin was utilized as the internal control.

Electrophoretic mobility shift assay (EMSA) of NF-κB

EMSA was performed using the LightShift™ chemiluminescent EMSA kit (Pierce Biotechnology) according to the manufacturer's protocol. Nuclear proteins were extracted from HRGECs with a nuclear extraction kit (Pierce Biotechnology). Extracted nuclear proteins were incubated with a biotin-labeled NF-κB probe (5'-AGT TGA GGG GAC TTT CCC AGG C-3') at room temperature for 20 min. Samples were separated by 6% non-denaturing polyacrylamide gel electrophoresis and transferred to a positively charged Nylon membrane (0.45 μm, Pierce Biotechnology).

Statistical analysis

All data were presented as mean ± SE. Data analysis were performed using One-way ANOVA, and the values of $P < 0.05$ were considered significant. All analyses were conducted with SPSS 17.0 statistical software (Chicago, Ill., USA).

RESULTS

Effects of high glucose on the kinetic activation of LCP

Firstly, cell surface deposition of MBL, C3 and MBL+C3 after exposure to high glucose (30 mmol/L) for various time periods demonstrated a time-dependent increase that peaked at 72 h ($P < 0.05$) (Figure 2A). The maximum increase of positive cells with fluorescence labeling of MBL, C3 and MBL+C3 was calculated as 13.23 ± 1.05, 3.80 ± 0.60 and 2.20 ± 0.40%, respectively at 72 h after stimulation with high glucose.

Secondly, cell surface deposition of MBL, C3 and MBL+C3 after exposure to glucose at various concentrations demonstrated a dose-dependent increase that peaked at 60 mmol/L ($P < 0.05$) (Figure 2B). The maximum increase of positive cells with fluorescence labeling of MBL, C3 and MBL+C3 was calculated as 17.93 ± 1.11, 6.27 ± 0.70 and 2.87 ± 0.40%, respectively at 60 mmol/L glucose. Our results (Figures 2A and B) demonstrated that high glucose stimulation *in vitro* played an important role in augmentation of complement activation on HRGECs.

Effects of exogenous rhuMBL on the kinetic activation of LCP

Firstly, cell surface deposition of MBL, C3 and MBL+C3 after treatment with exogenous rhuMBL (1 μg/ml) for various time periods increased in a time-dependent manner, peaked at 4 h and then decreased ($P < 0.05$) (Figure 3A). The maximum increase of positive cells with fluorescence labeling of MBL, C3 and MBL+C3 was calculated as 12.97 ± 0.80, 3.70 ± 0.50 and 2.10 ± 0.30%, respectively, at 4 h after treatment with exogenous rhuMBL.

Secondly, cell surface deposition of MBL, C3 and MBL+C3 after treatment with exogenous rhuMBL at various concentrations demonstrated a dose-dependent increase that peaked at 8 μg/ml ($P < 0.05$) (Figure 3B). The maximum increase of positive cells with fluorescence labeling of MBL, C3 and MBL+C3 was calculated as 84.10 ± 3.82, 19.80 ± 1.55 and 18.93 ± 1.30%, respectively at the highest exogenous rhuMBL concentration (8 μg/ml). These findings (Figures 3A and B) have confirmed that MBL is essential for the LCP activation following high glucose stimulation of HRGECs.

Inhibition experiments based on MAb 3F8

Flow cytometry

Compared with the MBL-Def group, cell-bound MBL, C3 and MBL+C3 deposition were significantly increased in the high-MBL group ($P < 0.01$). However, treatment with MAb 3F8 significantly inhibited MBL, C3 and MBL+C3 deposition on the cell surface ($P < 0.01$) (Figure 4). These results indicate that MAb 3F8 inhibited both MBL binding and C3 activation on the cell surface.

Immunofluorescence

Compared with the MBL-Def group, both MBL and C3 deposition stained strongly in the high-MBL group ($P < 0.01$) (Figure 5). Moreover, MBL staining significantly correlated with C3 deposition in the high-MBL group, suggesting LCP activation (Collard et al., 2000). Treatment with MAb 3F8 significantly attenuated both MBL and C3 deposition ($P < 0.01$) (Figure 6), which confirmed previous flow cytometry results.

Furthermore, cell surface MAC deposition in the high-MBL group stained more intensely compared with the MBL-Def group ($P < 0.05$). Treatment with MAb 3F8 significantly decreased MAC staining ($P < 0.05$) (Figure 7). These data demonstrated that MAb 3F8 inhibited MBL binding, C3 activation and MAC deposition.

Western blot

Because MBL is predominately synthesized in the liver, we used normal human hepatic tissue as the positive control, which was obtained from benign hemangiomas of resected livers. Western blot analysis demonstrated that cell-bound MBL deposition on HRGECs following addition of exogenous rhuMBL was attenuated by MAb 3F8 treatment ($P < 0.05$), whereas the MBL-Def group displayed no MBL deposition (Figure 8).

EMSA

DNA-binding activity of NF-κB was assessed by EMSA of

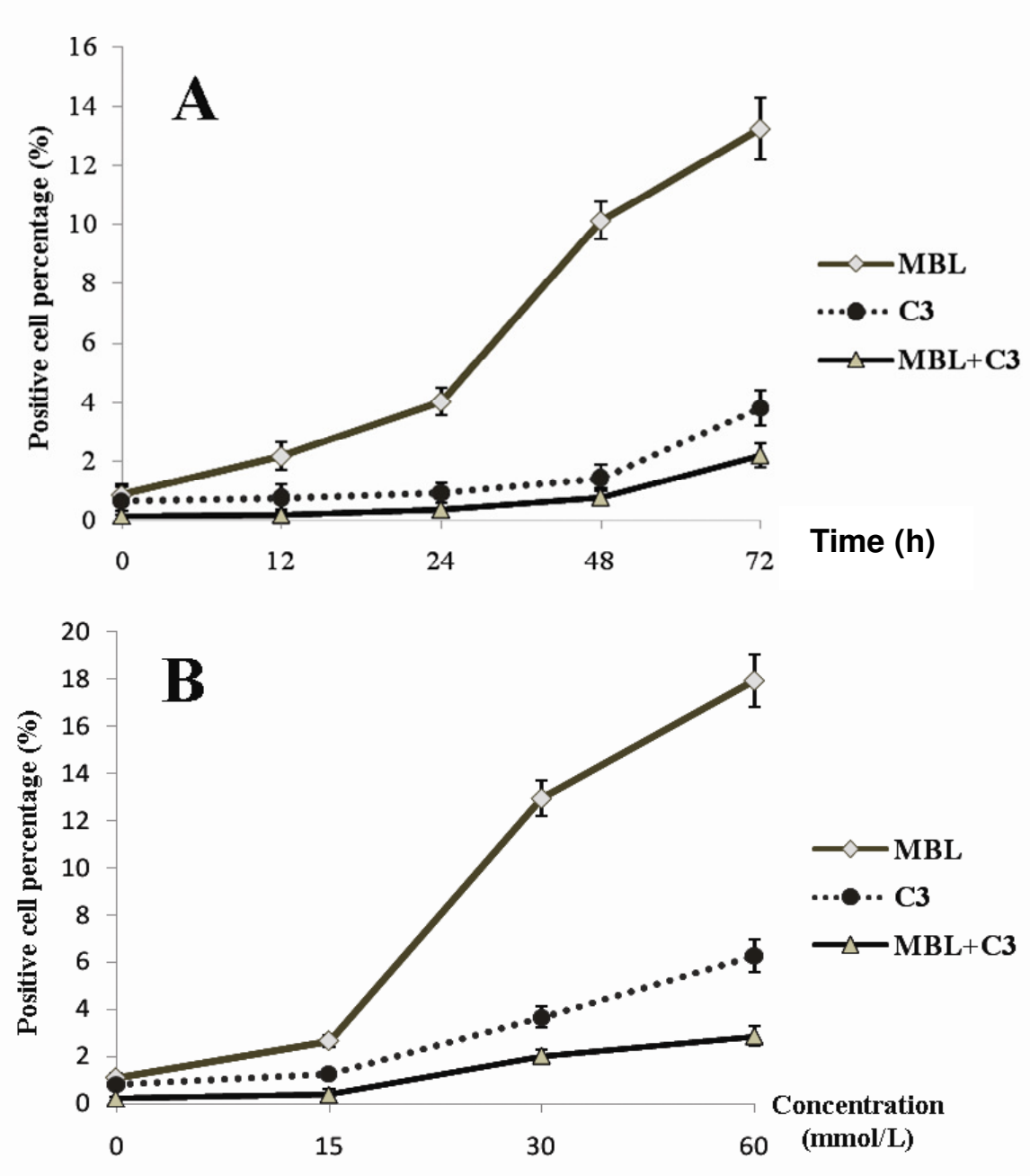

Figure 2. Time and dose effects of high glucose on the cell surface deposition of MBL, C3 and MBL+C3 by flow cytometry. A: The percentage of positive cells with fluorescence labeling of MBL, C3 and MBL+ C3 after exposure to high glucose (30 mmol/L) for various time periods (0, 12, 24, 48 or 72 h) increased in a time-dependent manner according to the graph, respectively ($P < 0.05$); B: The percentage of positive cells with fluorescence labeling of MBL, C3 and MBL+C3 after exposure to glucose at various concentrations (0, 15, 30 or 60 mmol/L) for 72 h increased in a dose-dependent manner according to the graph, respectively, ($P < 0.05$).

nuclear extracts from HRGECs. In contrast, a relatively weak NF-κB activation was observed in the MBL-Def group, whereas a strong nuclear activation of NF-κB was seen in cells from the high-MBL group ($P < 0.05$). In addition, the DNA-binding activity of NF-κB was significantly inhibited by MAb 3F8 treatment ($P < 0.05$) (Figure 9).

DISCUSSION

Proteinuria is a hallmark of DKD, and the glomerular endothelium is the first barrier to proteinuria in DKD. Specialized glomerular endothelial cells, which are located in the innermost part of glomerular filtration barrier, differ from other endothelial cells. Firstly, they have characteristic

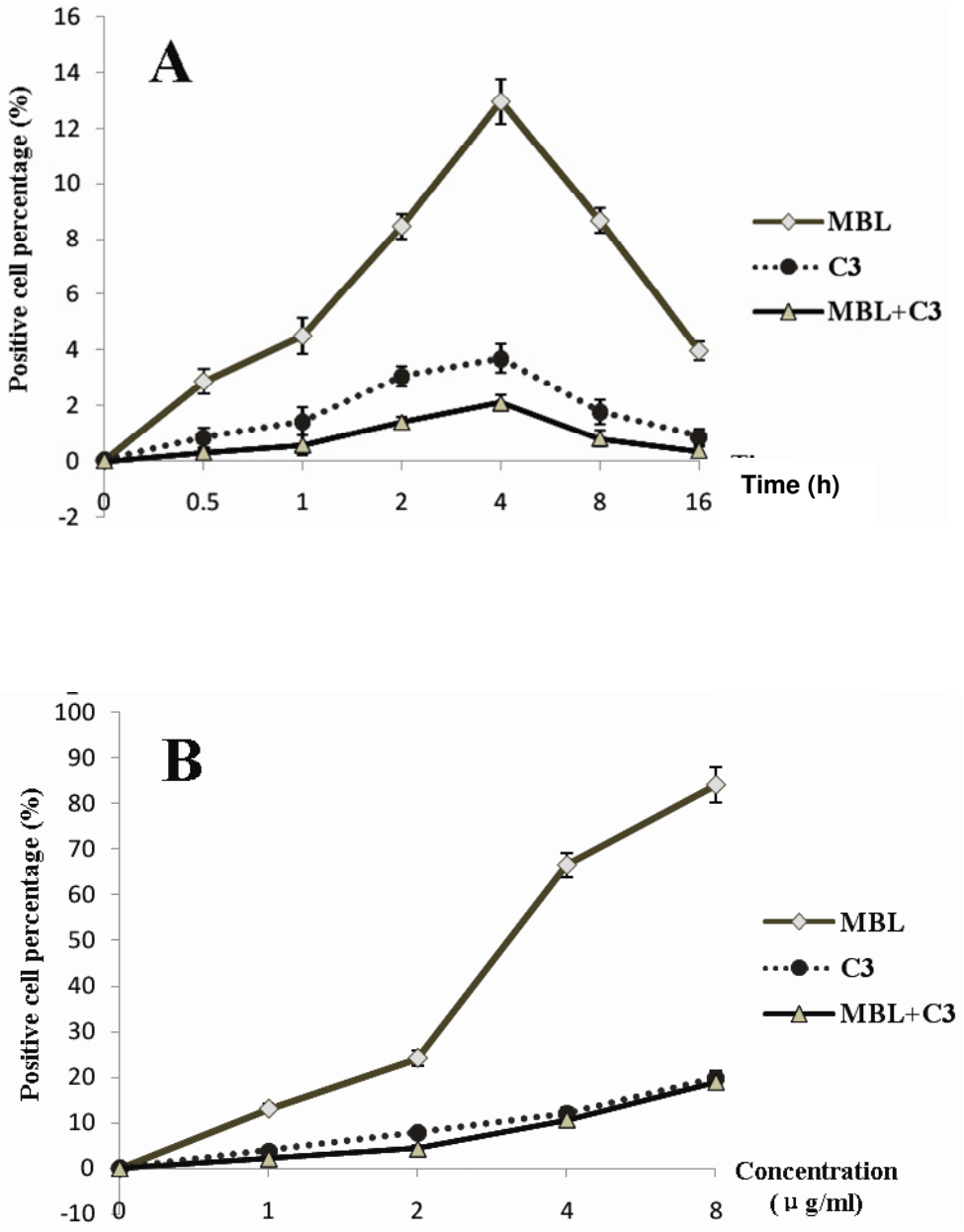

Figure 3. Time and dose effects of exogenous rhuMBL on the cell surface deposition of MBL, C3 and MBL + C3 by flow cytometry. A: The percentage of positive cells with fluorescence labeling of MBL, C3 and MBL+ C3 after treatment with exogenous rhuMBL (1 μg/ml) for various time periods (0, 0.5, 1, 2, 4, 8 or 16 h) increased in a time-dependent manner, peaked at 4 h and then decreased ($P < 0.05$); B: The percentage of positive cells with fluorescence labeling of MBL, C3 and MBL+ C3 after treatment with exogenous rhuMBL at various concentrations (0, 1, 2, 4 or 8 μg/ml) for 4 h increased in a dose-dependent increase manner according to the graph ($P < 0.05$), respectively.

fenestrations that serve to facilitate ultrafiltration (Sugimoto et al., 2003). Secondly, these cells are coated by endothelial glycocalyx, which comprises a surface layer of membrane-associated proteoglycans, glycosamino-glycans (GAG), glycoproteins, glycolipids and associated plasma proteins (Jeansson and Haraldsson, 2006). Glomerular endothelial cell injury has been considered to play a pivotal role in the mechanisms of proteinuria in DKD (Jefferson et al., 2008).

According to our experimental results, cell surface deposition of MBL, C3 and MBL+C3 after exposure to high glucose was increased in both a time- and dose-dependent manner. Under normal circumstances, serum MBL does not react with the host's own tissues (Malhotra

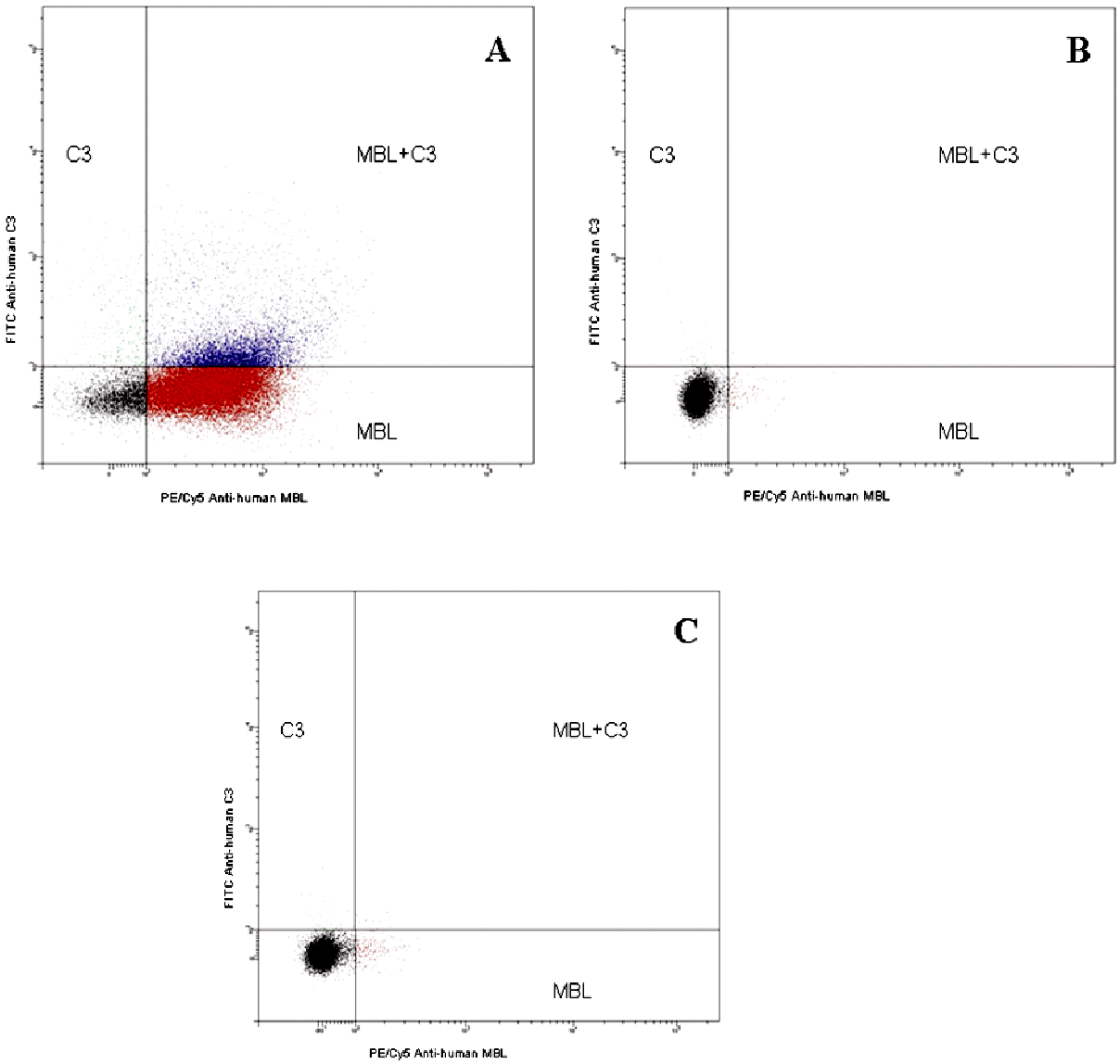

Figure 4. Flow cytometry assay of MBL, C3 and MBL+C3 deposition for the inhibition experiments. The percentage of positive cells with fluorescence labeling of MBL, C3 and MBL+C3 was observed to be significantly increased in the high-MBL group, which was significantly inhibited by MAb 3F8 treatment ($P < 0.01$). A: High-MBL group; B: MBL-Def group; C: MAb 3F8 group. (Red: Positive cells stained with MBL-PE/Cy5 alone; Green: Positive cells stained with C3-FITC alone; Blue: Positive cells doubled-stained with both MBL-PE/Cy5 and C3-FITC together; Black: Negative cells without MBL-PE/Cy5 or C3-FITC staining.)

et al., 1995). Our results demonstrated that high glucose stimulation of HRGECs could lead to increased MBL deposition and C3 activation. In correlation with our results, previous studies have shown that both MBL and C3 deposition colocalize on the cell surface, suggesting the activation of LCP (Collard et al., 2000). Evidence has indicated that excessive accumulation of non-enzymatic glycation products in endothelial cells plays an important role in the pathogenesis of chronic diabetic complications (Chibber et al., 1999). Hyperglycemia drives protein glycation, the non-enzymatic reaction of glucose with free α- or ε-amino groups in proteins that first result in the formation of a Schiff base or aldimine and then of the more stable ketoamine via the Amadori rearrangement (Qin et al., 2004).

The ketoamine moiety can also undergo further rearrangements, leading to the formation of advanced glycation end products (AGEs) (Nagaraj et al., 1996). The formation of AGEs has been shown to cause structural and functional changes in glycated proteins (Nawale et al., 2006), which may be recognized by the CRD of serum MBL. With prolonged exposure and increased glucose concentrations, the formation of protein glycation on the cell surface may increase. On the basis of these findings,

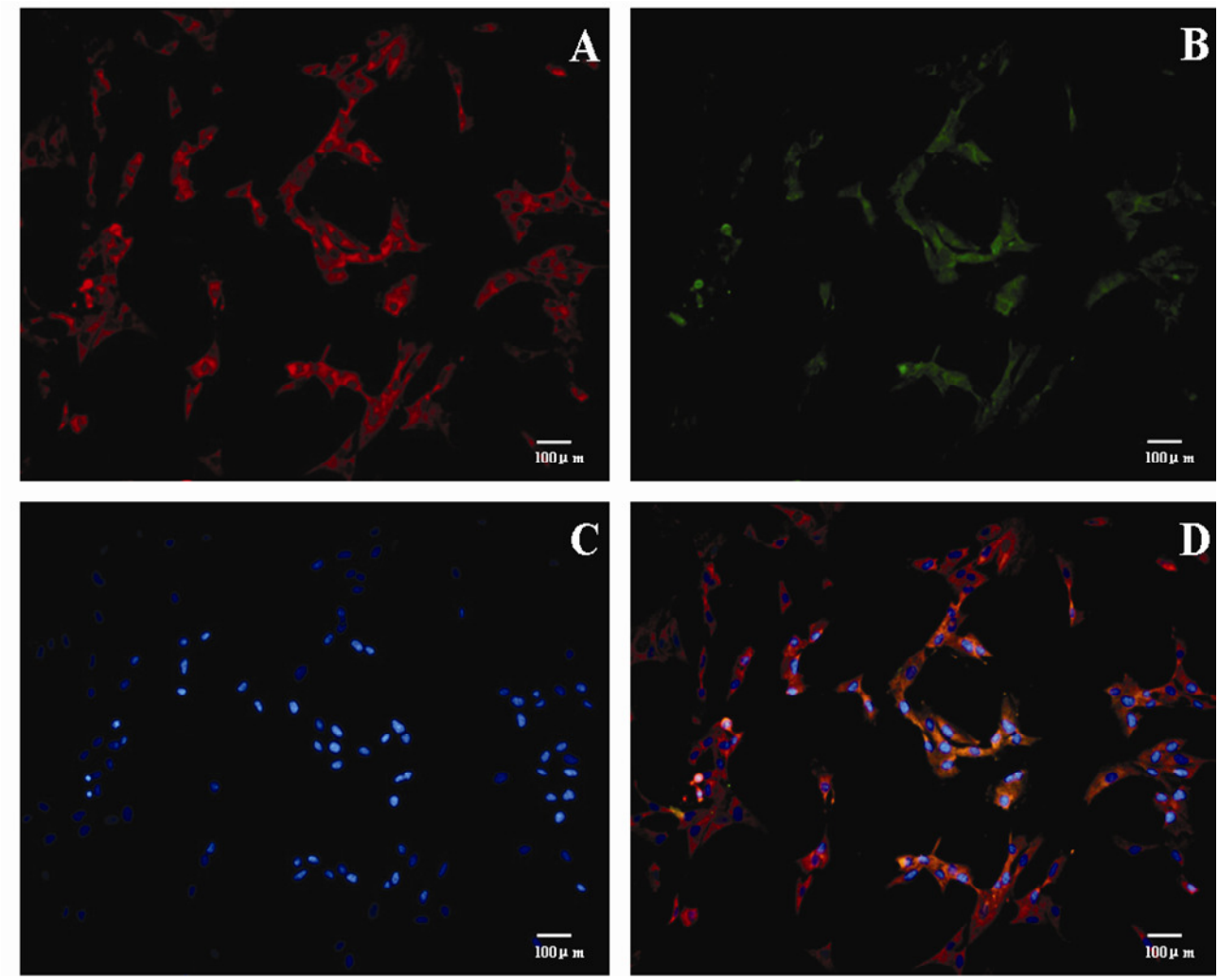

Figure 5. A series of IF images of MBL and C3 deposition in the high-MBL group by triple IF staining. A: MBL deposition stained with MBL-PE/Cy5 (red); B: C3 deposition stained with C3-FITC (green); C: Cell nuclei stained with DAPI (blue); D: Figures A, B and C were then merged and overlaid onto an image of triple IF staining for the same cells (200×).

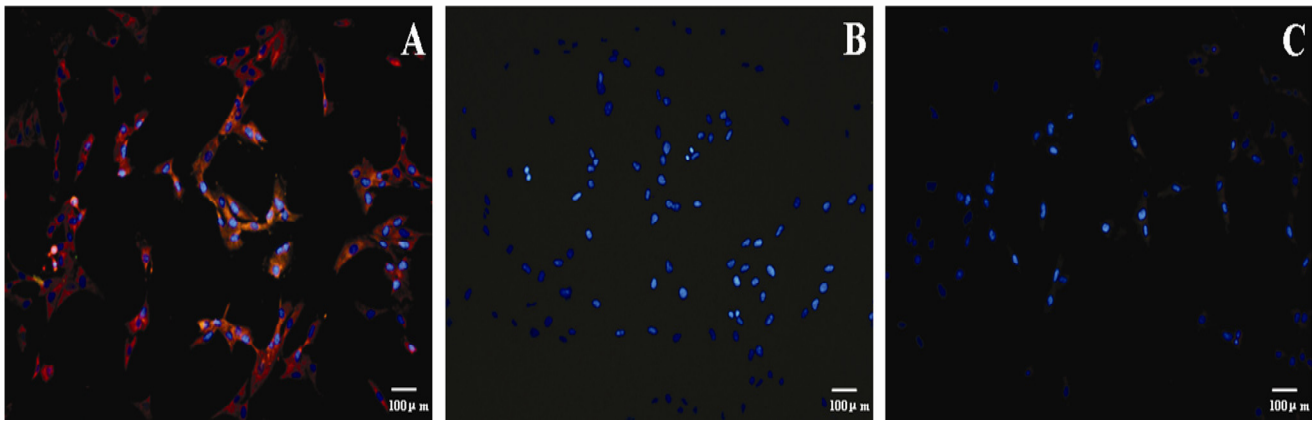

Figure 6. Triple IF staining of cell surface MBL and C3 deposition for the inhibition experiments. Merged images of triple IF staining for MBL and C3 deposition, stained with MBL-PE/Cy5 (red), C3-FITC (green) and DAPI (blue). Positive staining for MBL-PE/Cy5 and C3-FITC increased in cells of the high-MBL group, which was significantly attenuated by MAb 3F8 treatment ($P<0.01$). A: High-MBL group; B: MBL-Def group; C: MAb 3F8 group (200×).

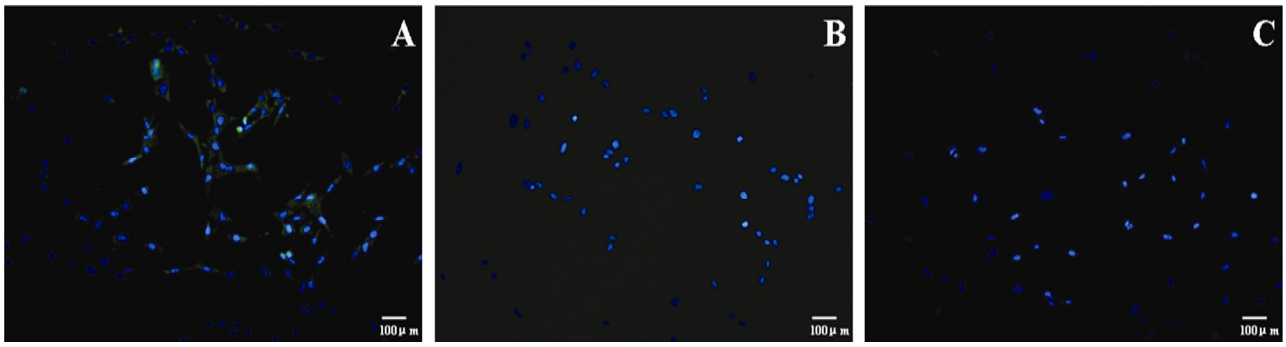

Figure 7. Double IF staining of cell surface MAC deposition for the inhibition experiments. Merged images of double IF staining for MAC deposition, stained with anti-C5b-9 and IgG-FITC (C5b-9 FITC, green), and DAPI (blue). Positive staining for C5b-9 FITC was observed in cells of the high-MBL group, which was significantly inhibited by MAb 3F8 treatment ($P < 0.05$). A: High-MBL group; B: MBL-Def group; C: MAb 3F8 group (200×).

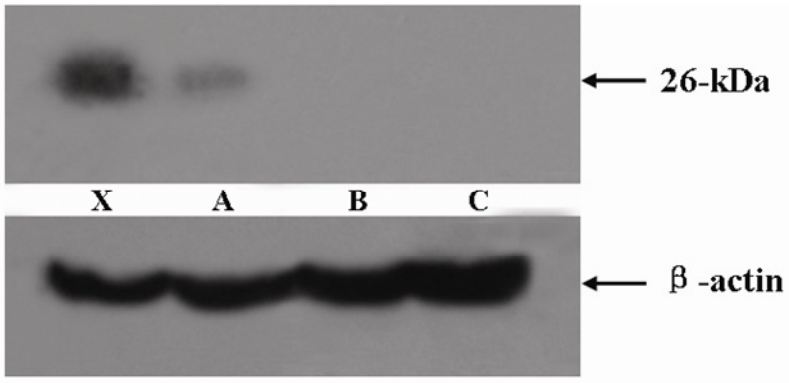

Figure 8. The cell surface protein deposition of MBL detected by Western blot analysis for the inhibition experiments. Increased MBL deposition was observed on cells of the high-MBL group, which was attenuated by MAb 3F8 treatment ($P < 0.05$). X: Positive control group (normal human hepatic tissue); A: High-MBL group; B: MBL-Def group; C: MAb 3F8 group.

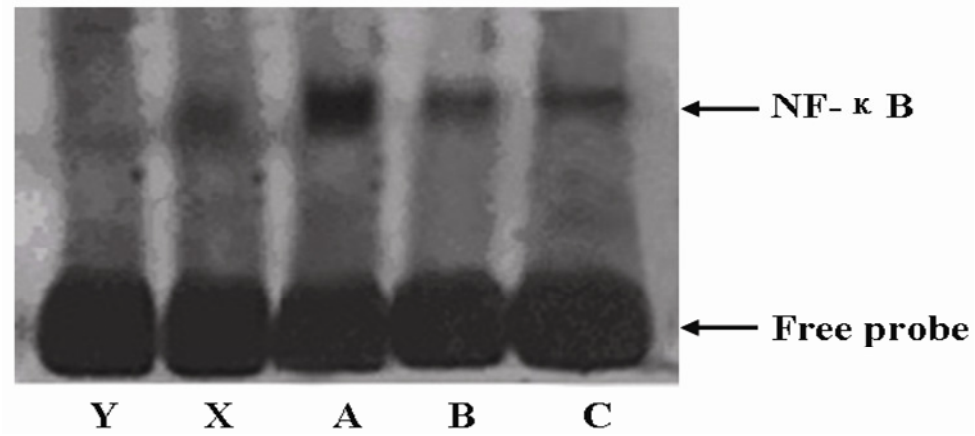

Figure 9. EMSA of NF-κB DNA-binding activity in HRGECs for the inhibition experiments. A strong nuclear activation of NF-κB was observed in cells of the high-MBL group, which was significantly inhibited by MAb 3F8 treatment ($P < 0.05$). Y: Negative control group (without nuclear protein); X: Positive control group; A: High-MBL group; B: MBL-Def group; C: MAb 3F8 group.

it could be hypothesized that MBL binding to enhanced glycated protein on the endothelial cell surface may initiate LCP activation stimulated by high glucose. Along these lines, the binding of MBL to fructosamines and the ensuing complement activation have recently been shown to be a potential mechanism linking enhanced glycation with complement activation in diabetes (Fortpied et al., 2010).

Additionally, cell surface deposition of MBL, C3 and MBL+C3 stimulated by exogenous rhuMBL was observed in time- and dose-dependent manner, peaking at 4 h and then decreasing. The median level of MBL in serum is 0.8 to 1 µg/ml, but approximately one-third of the population is < 0.5 µg/ml and about one tenth is < 0.1 µg/ml (Steffensen et al., 2000; Hansen et al., 2003). Mounting evidence suggested that serum MBL levels were significantly elevated in both type 1 and 2 diabetic patients with nephropathy (Hansen et al., 2004, 2006). MBL is predominately synthesized in the liver, and hepatic MBL expression may be chronically up-regulated as a consequence of low portal insulin levels in type 1 diabetic patients (Hansen et al., 2003). Glycation changes may induce increased MBL autoreactivity (Malhotra et al., 1995), which may explain why diabetic patients are more susceptible to the unfavorable effects of high MBL levels. However, MBL mRNA expression was not found in HRGECs stimulated by the high concentration of glucose and MBL according to our preliminary experiment. Thus, we speculated that elevated MBL levels may lead to augmented LCP activation following binding to enhanced glycated protein on the endothelial cell surface. With prolonged exposure to exogenous rhuMBL, the cell surface MBL, C3 and MBL+C3 deposition increased during the first 4 h, and then decreased, which may be associated with enhanced complement consumption through the LCP activation *in vitro* (Palarasah et al., 2010). In a recent large prospective study of 1564 patients with type 1 diabetes, elevated MBL levels above the median was significantly associated with the progression of renal disease (Hansen et al., 2010). Along these lines, increasing the concentration of exogenous rhuMBL further increased cell surface MBL deposition and complement activation.

Positive staining for MAC deposition was observed in cells of the high-MBL group, suggesting that LCP activetion stimulated by the high concentration of glucose and MBL lead to the formation of MAC. Complement activation and nonlytic MAC formation on endothelium has been shown to activate endothelial cells (Ward, 1996), and induce the release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), chemotactic factors such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), as well as adhesion molecules including P-selectin, E-selectin and intercellular adhesion molecule-1 (ICAM-1) (Acosta et al., 1996; Kilgore et al., 1996, 1997; Tramontini et al., 2002). These findings indicated that MAC-mediated inflammatory response may contribute to endothelial cell injury or dysfunction. As expected, our study also demonstrated increased NF-κB DNA-binding activity in the high-MBL group. These results were similar to others that have shown NF-κB activation by sublytic amounts of MAC (Viedt et al., 2000), suggesting that the MAC-dependent activation may lead to NF-κB activation of HRGECs. Mounting evidence suggested that NF-κB was of central importance in regulating inflammatory response for endothelial cells, and endothelial selective NF-κB blockade could significantly inhibit endothelial inflammation and activation (Xu et al., 2010). Moreover, recent data demonstrated that high glucose-induced nascent nephron apoptosis was mediated via activation of NF-κB pathway (Chen et al., 2011). Thus, MAC-mediated effects, including NF-κB activation, may be implicated in the pathogenesis of endothelial cell inflammation and injury after stimulation of the high concentration of glucose and MBL.

Finally, we used the functional inhibitory MAb 3F8 antibody to block LCP activation of HRGECs. According to our results, MAb 3F8 could significantly inhibit MBL deposition and subsequent LCP activation stimulated by the high concentration of glucose and MBL *in vitro*. Furthermore, the suppression of NF-κB activation was observed by MAb 3F8 treatment. Therefore, a novel treatment strategy might be developed for DKD based on the inhibition of LCP activation. MAb 3F8 is one of the most useful inhibitory antibodies against human MBL, which preferentially binds to MBL with high affinity. The inhibition of MBL binding by MAb 3F8 is most likely due to its recognizing and binding to a discontinuous epitope in the hinge region within the CRD of MBL, which may induce an overall conformational change within MBL and subsequently disrupt its ability to bind to its substrates (Zhao et al., 2002).

In summary, our data provide a new insight that high glucose and MBL play an important role on the LCP activation of HRGECs, which may be associated with enhanced protein glycation and elevated MBL levels. Moreover, MAC-mediated effects, including NF-κB activation, may be implicated in the pathogenesis of endothelial cell inflammation and injury stimulated by the high concentration of glucose and MBL. Inhibition experiments support that MAb 3F8 may represent a novel potential therapeutic strategy to block LCP activation on HRGECs.

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