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Accelerated Glomerular Cell Senescence in Experimental Lupus Nephritis

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Background: The aim of this study was to determine whether senescence in renal glomeruli is involved in lupus nephritis (LN); the expression of senescence-associated β-galactosidase (SA-β-Gal) and its association with glomerular lesions were investigated in a mouse model of LN.

Material/Methods: Eighteen MRL/lpr mice with severe proteinuria were randomly divided into 2 equal groups and intraperitoneally injected with dexamethasone (DEX) or saline; 4 age-matched mice with mild proteinuria served as controls. Serum creatinine and urinary protein levels were analyzed, and kidney histological changes were observed by periodic acid–Schiff and Sirius Red staining. SA-β-Gal was detected via histochemistry. Glomerular expression of collagen IV, α-SMA, and nephrin was analyzed by immunohistochemistry, and glomerular complement C3 deposition was tested by immunofluorescence. The relationships between SA-β-Gal expression and renal function or glomerular lesion markers were determined by Spearman's correlation analysis.

Results: Mice with severe proteinuria exhibited glomerular segmental sclerosis and endothelial cell proliferation. DEX administration suppressed these lesions but had no significant effect on 24-hour urinary protein levels. The elevated glomerular expression of SA-β-Gal in proteinuric mice was attenuated by DEX treatment. In addition, DEX treatment markedly downregulated glomerular C3 deposition and collagen IV and α-SMA expression, while significantly increasing nephrin expression. Furthermore, SA-β-Gal expression was positively correlated with urinary protein levels and expression of α-SMA.

Conclusions: Accelerated senescence of glomerular cells may contribute to glomerular injury in LN.

MeSH Keywords: **Cell Aging • Dexamethasone • Glomerulonephritis • Lupus Nephritis • Podocytes**

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Background

Lupus nephritis (LN), characterized by glomerular deposition of immune complex followed by a sustained inflammatory response, is the most common manifestation and the main cause of mortality in systemic lupus erythematosus (SLE) [1]. Glomerular pathological changes in LN are diverse, mainly including the proliferation of mesangial cells, thickening of the basement membrane, damage or loss of podocytes, and segmental sclerosis, leading to increased glomerular permeability and abnormal proteinuria [2,3]. The immune mechanisms involved in glomerular injury are well documented, and immunosuppressive agents improve long-term outcomes in LN patients. Even with proper treatment from a nephrologist, 40% of patients with LN develop some degree of renal dysfunction [4]. This reflects our incomplete knowledge of the pathogenic mechanisms of this disease. A growing body of evidence suggests that non-immune mechanisms of glomerular injury should be considered for adequate treatment of LN [5].

Cellular senescence is defined as an irreversible cell growth arrest with abnormal phenotype and function. Senescent cells exhibit the upregulated expression of senescence markers such as senescence-associated β-galactosidase (SA-β-Gal), promoting the accumulation of negative cell cycle regulator proteins and the senescence-associated product lipofuscin. Cell senescence occurs during natural aging in a telomere-dependent manner or can be induced as physiological senescence driven by DNA damage and oxidative stress and mediated by cyclin-dependent kinase (CDK) inhibitors [6-8]. Recently, cell senescence has been implicated in glomerular lesions that develop under disease conditions [9]. In diabetic nephropathy, glomerular cell senescence induced by high glucose positively correlates with proteinuria, which is mainly mediated by mitochondrial dysfunction and elevated reactive oxygen species [10]. In addition, the kidneys of patients with membranous nephropathy, focal segmental glomerulosclerosis (FSGS), and minimal change disease show upregulation of the senescence marker $p16^{INK4A}$ in the glomeruli. The degree of glomerular cell senescence is also associated with renal disease progression [11]. However, whether cell senescence is involved in glomerular injury in LN remains unknown.

In the present study, to test the hypothesis that cell senescence in glomeruli is involved in LN, we assessed the glomerular expression of SA-β-Gal in spontaneous lupus-prone MRL/lpr mice and analyzed its association with renal function and glomerular lesion marker expression.

Material and Methods

Animal model

Female MRL/lpr mice spontaneously develop systemic autoimmunity and immune-complex glomerulonephritis with aberrant T cell proliferation as a result of a *Fas* gene deficiency. This strain is useful as a model for determining the pathogenic mechanisms of SLE and LN [12]. In the present study, female MRL/lpr mice (12 weeks old; 33±2 g) were supplied by the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Animals were housed in a special pathogen-free environment (25°C, normal 12-h light/12-h dark cycle) at the Animal Center of Guangdong Medical University. The mice had unrestricted access to standard food and sterilized water supplied by the facility. Eighteen MRL/lpr mice with severe proteinuria (concentration above 1 mg/ml) were randomly divided into 2 equal groups that received dexamethasone (DEX) treatment (DEX group) or equivalent volumes of saline (LN group). DEX (1.5 mg/kg body weight) was administrated by intraperitoneal injection 3 times a week for 4 weeks. Four age-matched MRL/lpr mice with mild proteinuria (concentration less than 0.3 mg/ml) were included as a control (control group) and were injected with saline for 4 weeks. After treatment, all mice were euthanized, and blood and kidney tissue samples were collected for subsequent tests. All the procedures were approved by the Animal Experimentation Ethics Committee of Guangdong Medical University and conformed to approved guidelines.

Detection of serum creatinine and 24-h urinary protein

Blood was collected from the hearts of mice. Serum was isolated by centrifuging in a serum-separating tube, and then serum creatinine was measured using a colorimetry kit (C011-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol. All mice were placed in metabolic cages for 24-h urinary protein excretion, and the urinary protein concentration was measured by the Bradford assay (Cat. 500-0205, Bio-Rad, Hercules, CA, USA) as described previously [13]. Levels of 24-h urinary protein were calculated by determining the concentration and multiplying by urine volume.

Morphological observation

Kidney samples were collected, fixed in Carnoy's solution at 25(±2)°C overnight, dehydrated in ethanol, and embedded in paraffin following standard procedures. Changes in renal morphology were detected in tissue sections (3-μm) using the periodic acid–Schiff (PAS) method and Sirius Red staining. Glomerulosclerosis was assessed using a semi-quantitative scale as described by Cao et al. [14]. In brief, 12 PAS-stained random glomeruli per section were examined and graded as follows: 0, normal; 1, mesangial expansion and slight glomerular

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damage involving <25% of the glomerulus; 2, mild sclerosis involving 25–50% of the glomerulus; 3, moderate sclerosis involving 50–75% of the glomerulus; and 4, severe sclerosis involving >75% of the glomerulus.

SA-β-Gal staining

SA-β-Gal staining was performed using the senescence β-galactosidase staining kit (C0602, Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, kidney samples were fixed using the paraformaldehyde/sucrose method and embedded in OCT medium for cryostat sectioning. Then, the 5-μm sections were washed 3 times in phosphate- buffered saline (PBS) and incubated in freshly prepared SA-β-gal staining solution (pH=6.0) for 16 h at 37°C. To identify the type of SA-β-Gal-positive glomerular cells, tissue sections were counterstained with silver staining and then mounted with neutral balsam. Double staining SA-β-Gal and podocyte marker nephrin was performed to identify the origin of glomerular senescent cells. The expression of nephrin was detected by immunohistochemistry as described below in frozen sections following SA-β-Gal staining.

For each sample, 10 fields were collected at a magnification of 400× under a light microscope (Leica DMI 3000B, Leica Microsystems, Wetzlar, Germany). Semi-quantitative analysis of glomerular SA-β-gal-positive cells was performed using the following scale (0–3): 0=negative staining, 1=rare accidental positive staining, 2=focal positive staining, and 3=diffuse positive staining [15].

Immunohistochemistry

For immunohistochemical analysis, paraffin-embedded sections were deparaffinized and rehydrated. For antigen retrieval, sections were autoclaved in 0.01 M citrate buffer solution (pH=6.0) for 3 min. Endogenous peroxidase was quenched with 3% hydrogen dioxide for 30 min. After PBS washing, sections were incubated with 5% bovine serum albumin (BSA) to block non-specific interactions before incubation with rabbit polyclonal anti-α-SMA (ab124964, Abcam, Cambridge, UK), rabbit polyclonal anti-collagen IV (ab6586, Abcam), or goat polyclonal anti-nephrin (AF3159-SP, R&D Systems, Minneapolis, MN, USA) overnight at 4°C. Then, sections were incubated with goat anti-rabbit immunoglobulin (Ig)G-horseradish peroxide (HRP) (ZB2301, Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) for α-SMA and collagen IV or donkey anti-goat IgG-HRP (A0181, Beyotime Biotechnology, Shanghai, China) for nephrin. diaminobenzidine (DAB) solution (ZLI-9017, Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) was added to slides for the detection of antibodies labeled with HRP. Negative controls were performed by replacing primary antibodies with PBS. All slides were counterstained with hematoxylin solution and then mounted with neutral balsam. For each sample, 10–15 fields were collected at 400× magnification under a light microscope. The positive areas (%) for collagen IV, α-SMA, and nephrin were measured in glomeruli using Image J software (NIH, Bethesda, MD, USA).

Immunofluorescence

For detection of complement C3, 5-μm frozen kidney sections were incubated with goat anti-mouse C3-FITC-conjugated antibody (GC3-90F-Z, ICL, Atlanta, GA, USA) and then mounted with anti-fade mounting medium (P0126, Beyotime). For each sample, 10 fields were observed at 400× magnification using a TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). Semi-quantitative analysis of glomerular complement C3 deposition was performed using the following scale (0–3): 0=negative staining, 1=barely visible at high magnification, 2=moderately visible, and 3=clearly visible [16].

Statistical analysis

The data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA) and SPSS software 17.0 (IBM, Armonk, NY, USA). The *t* test was used for statistical evaluation of the data, which are shown as mean ± standard error of the mean (SEM). Relationships between 2 sets of variables were determined by Spearman's rank correlation coefficient, and comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. $P<0.05$ was regarded as a statistically significant difference.

Results

Glomerular pathological changes and renal dysfunction in LN mice were suppressed by DEX treatment

Pathological alterations in the glomeruli were assessed by microscopic examination of sections with PAS and Sirius Red staining. As shown in Figure 1A, several glomeruli with diffuse endothelial cell proliferation, tuft-to-capsule adhesion, and segmental sclerosis were observed among mice in the LN group. In contrast, there was minimal glomerular injury among control group mice. Moreover, DEX administration markedly inhibited the glomerular pathological changes observed in LN group mice (Figure 1A, 1B). LN group mice, with severe urinary protein excretion, exhibited higher serum creatinine levels than the control group mice, whereas DEX treatment attenuated the increase in serum creatinine in LN mice. However, these differences in serum creatinine were not significant among the 3 groups (Figure 1C–1E). Of note, 1 mouse with higher concentration of urinary protein (8.196 mg/ml) in

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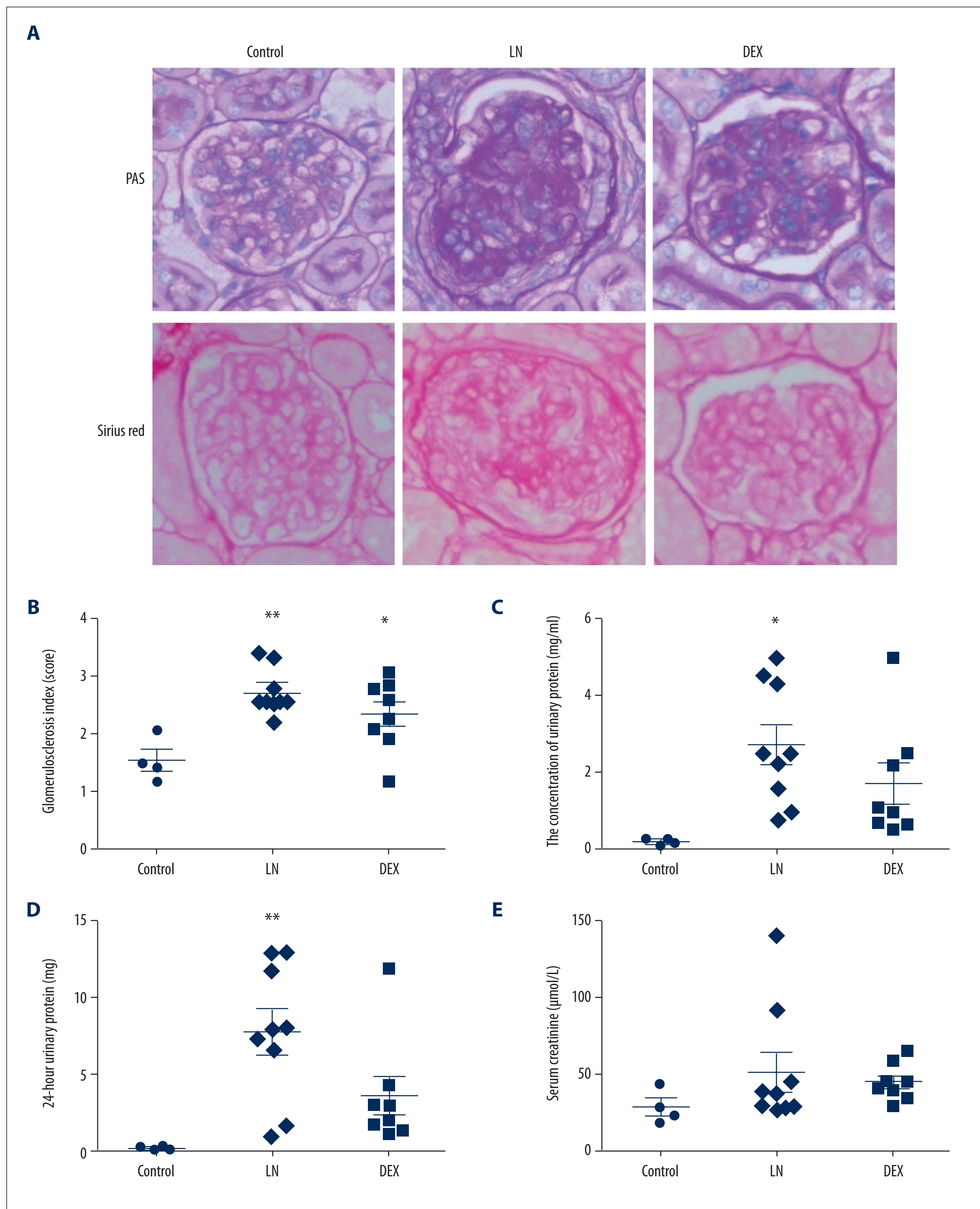

Figure 1. Evaluation of renal function and pathological changes. (**A**) Morphological observation by PAS and Sirius Red staining. Magnification: 400×. (**B**) Glomerulosclerosis index. (**C**) Concentrations of urinary protein. (**D**) Levels of 24-h urinary protein. (**E**) Serum creatinine levels. Each bar represents the mean ±SEM. * *P*<0.05 and ** *P*<0.01 compared with control group mice; n=4 in control group, n=9 in LN group and n=8 in DEX group. Circles – control group; Diamond – LN group; Squares – DEX group.

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the DEX group was considered as an outlier in SPSS analysis and was removed from subsequent analysis.

Increase in glomerular expression of SA-β-Gal in LN mice was attenuated by DEX administration

The expression of neutral β-galactosidase (pH=6.0) activity is widely used as a characteristic biomarker for the detection of senescent cells [17]. To explore whether senescent cells were present in the glomeruli of LN mice, frozen sections were stained using a senescence β-galactosidase staining kit. As shown in Figure 2, typical senescent cells are positive for blue pigment. By counterstaining with silver stain, we observed that the glomerular SA-β-Gal signal was predominantly confined to the podocytes (Figure 2A). Podocytes have been further proven to be the main cell type of glomerular senescent cells by a double staining of SA-β-Gal and nephrin (blue-stained senescent cells co-located with the brown signal of nephrin). Other glomerular SA-β-Gal-positive cells were mostly endothelial cells and mesangial cells (Figure 2C). Of note, SA-β-Gal staining was markedly higher in glomerular cells in the LN group mice than in those in control group mice. In contrast, DEX treatment decreased the expression of SA-β-Gal in the glomeruli of LN group mice (Figure 2B). These data indicate that DEX suppresses cellular senescence in glomeruli in LN mice.

DEX treatment downregulated glomerular expression of α-SMA and collagen IV and C3 deposition but upregulated nephrin expression in glomeruli

As complement C3 is the common point connecting all 3 pathways in complement activation, glomerular deposition of C3 was detected by direct immunofluorescence to assess complement activation. As shown in Figure 3Aa–c, 3B, C3 deposition was significantly increased in the glomeruli of LN mice compared to that in control mice, while DEX administration attenuated the increased C3 deposition.

To further assess glomerular lesions, we examined the typical pathological markers associated with LN. The expression of the extracellular matrix proteins collagen IV and α-SMA is elevated during glomerular sclerosis, and these proteins are therefore often used as markers to indicate the progression of glomerular sclerosis. In contrast, loss of nephrin expression in glomeruli suggests injury to podocytes, the crucial cells constituting the glomerular filtration barrier. The results of immunohistochemical staining showed that the glomerular expression of α-SMA (Figure 3Ae) and collagen IV (Figure 3Ah) was markedly enhanced, primarily in the mesangial area; however, nephrin (Figure 3Ak) expression was significantly reduced in LN mice compared to levels in control mice (Figure 3Ad, g, j). These changes in the expression levels of α-SMA, collagen IV, and nephrin in the glomeruli were reversed following DEX administration (Figure 3Af, i, l, 3C).

Expression of SA-β-Gal was positively correlated with urinary protein levels, expression of collagen IV and α-SMA, and C3 deposition

Spearman's correlation tests were performed to assess the relationship between the expression of SA-β-Gal and other variables. Correlation analysis showed that glomerular SA-β-Gal expression was positively correlated with the concentration of urinary protein ($r=0.5861$, $P<0.01$) and 24-h urinary protein ($r=0.4782$, $P<0.05$), whereas no association was observed between SA-β-Gal expression and serum creatinine ($r=-0.1287$, $P>0.05$; Figure 4A, B, G). Moreover, SA-β-Gal expression was strongly correlated with expression of α-SMA ($r=0.4717$, $P<0.05$) in glomeruli (Figure 4C). In contrast, glomerular expression of SA-β-Gal was not directly associated with the expression of the podocyte injury marker nephrin ($r=-0.1189$, $P>0.05$), collagen IV ($r=0.4107$, $P>0.05$), or with C3 deposition ($r=0.3778$, $P>0.05$, Figure 4D–4F).

Discussion

In the present study, we showed that kidneys from LN mice with severe proteinuria displayed accelerated glomerular expression of SA-β-Gal, correlating with glomerular lesion markers. Additionally, DEX administration inhibited the expression of SA-β-Gal, consistent with the downregulation of glomerular lesion markers. Our results suggest that accelerated senescence in glomeruli contribute to the progression of LN.

In this study, we used lupus mice with varying degrees of proteinuria to mimic lupus patients with varying degrees of renal damage. Lupus mice with severe proteinuria (concentrations above 1 mg/ml) were used to mimic lupus patients with severe renal damage. Due to the high heterogeneity among lupus mice, we used those with urinary protein concentrations less than 0.3 mg/ml as a control group to mimic lupus patients with mild renal damage.

It is well known that glomerular lesions in LN are triggered by the deposition of immune complex and subsequent complement activation. Complement activation products not only recruit inflammatory cells to induce glomerular cell injury by releasing inflammatory cytokines, but also cause intrinsic cell damage directly through the formation of membrane attack complexes [18]. All injuries to intrinsic cells induced by complement activation contribute to the glomerular lesion, but the pathogenesis is still unclear. Recently, cellular senescence has been reported to play a causal role in the pathogenesis of glomerular diseases [19].

β-galactosidase is a lysosomal hydrolase that is highly expressed in renal proximal tubular epithelial cells. Increased

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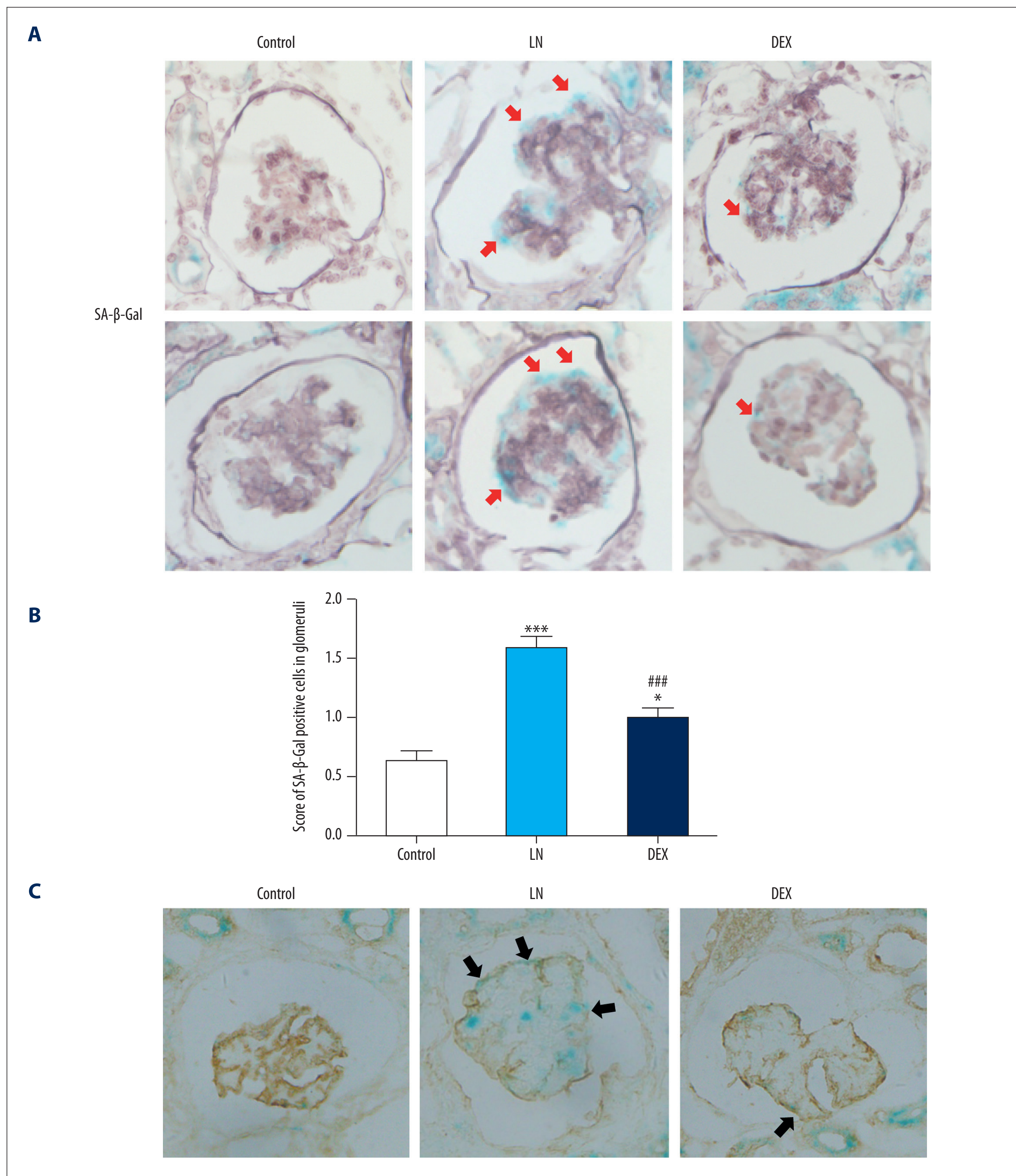

Figure 2. Glomerular expression of SA-β-Gal. (**A**) Representative image of SA-β-Gal staining counterstained with silver staining in control, LN, and DEX groups. Magnification: 400×. Red arrows indicate positive SA-β-Gal expression in podocytes. (**B**) Quantitative analysis of SA-β-Gal expression. (**C**) Double staining of SA-β-Gal and nephrin in control, LN, and DEX groups. Magnification: 400×. Black arrows indicate positive double staining of SA-β-Gal and nephrin. Each bar represents the mean ±SEM. * $P<0.05$ and *** $P<0.001$ compared with control group mice, ### $P<0.001$ compared with LN group mice; n=4 in control group, n=9 in LN group and n=8 in DEX group.

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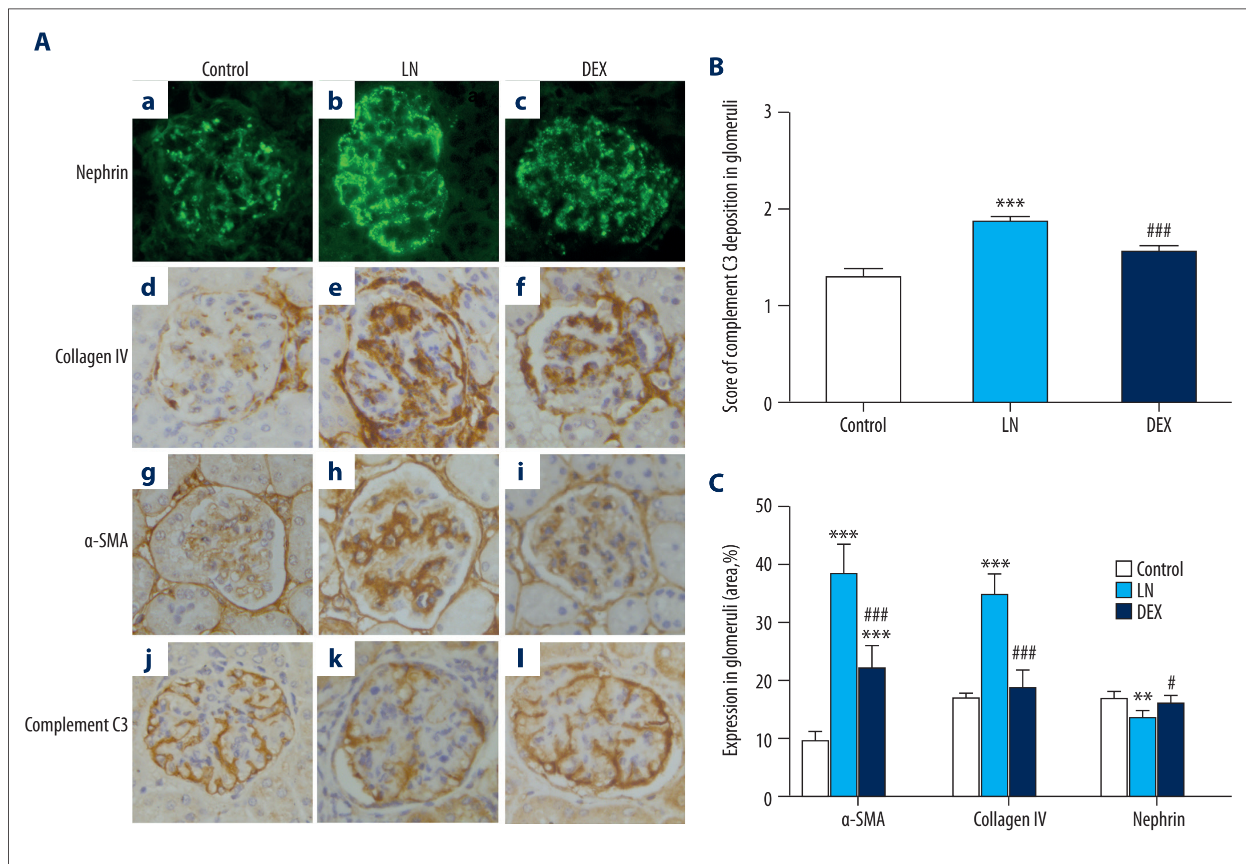

Figure 3. Immunohistochemical and immunofluorescent analyses. (**A**) Representative image of immunohistochemical and immunofluorescent staining in control, LN, and DEX groups. (**a–c**) Immunofluorescent analysis of C3 deposition. (**d–f**) Immunohistochemical analysis of α-SMA. (**g–i**) Immunohistochemical analysis of collagen IV. (**j–l**) Immunohistochemical analysis of nephrin. Magnification: 400×. (**B**) Quantitative analysis of C3 deposition. (**C**) Quantitative analysis of α-SMA, collagen IV, and nephrin expression. Each bar represents the mean ± SEM. ** *P*<0.01, *** *P*<0.001 compared with control group mice. # *P*<0.05, ### *P*<0.001 compared with LN group mice; n=4 in control group, n=9 in LN group and n=8 in DEX group.

urinary excretion of β-galactosidase can especially reflect the early tubular injury [20,21]. Unlike urinary β-galactosidase that were detected at pH 3.8, increased SA-β-Gal activity that was detected *in situ* but not in urine at pH 6.0 predicts renal cell senescence [11]. In our study, we found that glomerular cells in LN mice with severe proteinuria dramatically upregulated the senescence marker SA-β-Gal, accompanied by increased deposition of central complement component C3 in the glomerulus. Elevations in both SA-β-Gal expression and C3 deposition were attenuated by DEX administration. From the perspective of pathology, inflammatory cell-derived IL-6, IL-1, MCP-1, and other cytokines induced by complement activation may mediate glomerular-resident cell senescence via the NF-κB pathway [22]. Another possible explanation is that complement C3 and its signaling pathway directly promote the senescence-like phenotype, which has been shown in previous studies [23]. Thus, our data indicate that complement deposition and activation, and the subsequent inflammatory response in the glomerulus, are important upstream events that trigger glomerular-resident cell senescence.

In addition, our study showed that podocytes were the predominant cells expressing the senescence marker SA-β-Gal in LN mice with severe proteinuria. Moreover, the essential podocyte protein nephrin was downregulated in LN mice with severe proteinuria, but its expression was partially restored by DEX treatment. However, there was no relationship between SA-β-Gal and nephrin expression. It is well documented that lack of nephrin expression impairs the slit diaphragm, a crucial structural component of the glomerular filtration barrier, and causes proteinuria. In addition, nephrin dislocation from the podocyte plasma membrane to an extracellular site disrupts the integrity of the glomerular slit diaphragm [24]. Our findings indicate that podocyte expression of SA-β-Gal may be correlated with nephrin dislocation but not its expression. However, we did not assess glomerular nephrin dislocation due to limitations in research methods.

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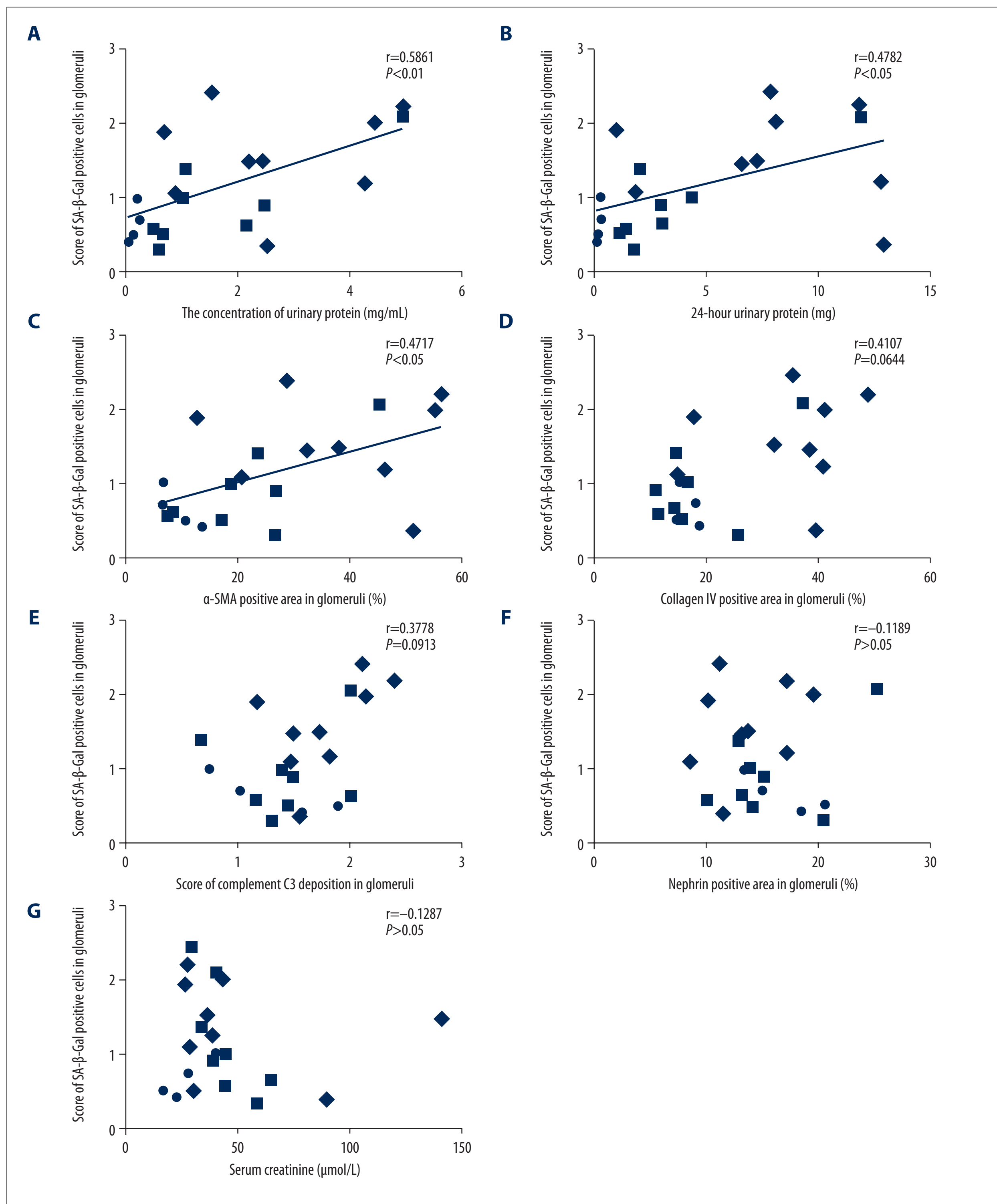

Figure 4. Spearman's correlation analyses. (**A**) Relationship between SA-β-Gal expression and concentration of urinary protein. (**B**) Relationship between SA-β-Gal expression and 24-h urinary protein. (**C**) Relationship between expression levels of SA-β-Gal and α-SMA. (**D**) Relationship between expressions of SA-β-Gal and Collagen IV. (**E**) Relationship between SA-β-Gal expression and C3 deposition. (**F**) Relationship between expression levels of SA-β-Gal and nephrin. (**G**) Relationship between SA-β-Gal expression and serum creatinine. n=4 in control group, n=9 in LN group, and n=8 in DEX group. Circles – control group; Diamond – LN group; Squares – DEX group.

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The accelerated senescence of glomerular cells plays an indispensable role in the progression of LN, and significantly contributes to glomerular lesions. In this study, we found that glomerular SA-β-Gal expression was positively associated with α-SMA in the mesangial area, but less relationship with the extracellular matrix components collagen IV was not statistically significant due to our smaller sample size. Our data indicate that SA-β-Gal expression is tightly associated with glomerular sclerosis in LN. Consistent with this, it has been shown that renal intrinsic cells gradually express senescence markers (such as SA-β-Gal and cell cycle inhibitor $p16^{INK4A}$) in parallel with glomerular sclerosis, tubular atrophy, interstitial fibrosis, and proteinuria in human and glomerular diseases, including diabetic nephropathy [11], IgA nephropathy [25], nephrotic syndrome [11], polycystic kidney disease [26], and nephronophthisis [27]. Mechanistically, senescent cells exert a senescence-associated secretory phenotype (SASP) that releases massive amounts of pro-inflammatory cytokines (e.g., IL-6, IL-1β, MCP-1, and IL-8) through paracrine mechanisms [28], contributing to glomerular inflammation. Furthermore, senescent cells produce aberrant growth factors such as TGF-β [29]. In response to the inflammatory cytokines and growth factors produced by senescent cells, glomerular mesangial cells become activated, proliferate, and acquire myofibroblast-like phenotype with strong expression of α-SMA. Mesangial cells with this phenotype mainly secrete a large number of extracellular matrix proteins (such as collagen IV), which cause glomerular sclerosis. Additionally, a possible explanation for the strong correlation between SA-β-Gal expression and proteinuria is that damage to the glomerular filtration barrier induced by senescent cell-driven inflammation and sclerosis causes abnormal urinary protein excretion. However, SA-β-Gal expression was not correlated with serum creatinine levels in LN mice.

Our study has some shortcomings. DEX administration significantly improved glomerular inflammatory and fibrotic changes but did not significantly reduce proteinuria. First, our finding that DEX administration did not significantly suppress urinary protein excretion may result from our limited sample size, as evidenced by the low power of the tests (data not shown). Second, although the decrease in nephrin expression, which impairs the slit diaphragm and leads to proteinuria in LN mice, was partially ameliorated by DEX treatment, nephrin distribution may not be preserved, but we did not assess this due to limitations in research methods. Nephrin dislocation disrupts the integrity of the glomerular slit diaphragm, which also results in proteinuria [24]. Third, in addition to podocyte injury, impairment of tubular reabsorption also causes urinary protein excretion. Moreover, our findings need to be proven in subsequent studies of human LN.

Conclusions

Taken together, our results indicate that accelerated cell senescence is involved in glomerular injury and may contribute to the progression of LN.

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Conflicts of interests

None.

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