

THE IMMUNOEXPRESSION OF GLOMERULAR NF- κ B IN PROTEINURIC PATIENTS WITH PROLIFERATIVE AND NON-PROLIFERATIVE GLOMERULOPATHIES

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Recent studies suggest that NF- κ B activation plays an essential role in the activation of mesangial cells and macrophages through the transcriptional induction of inflammatory mediators of glomerular inflammation and injury. The aim of the present study was to determine, using an image analysis system, glomerular immunoeexpression of NF- κ B (nuclear translocation of p65) in proteinuric patients with proliferative and non-proliferative glomerulopathies. Thirty-six proteinuric patients with idiopathic proliferative glomerulopathies (PG group) and 28 proteinuric participants with non-proliferative glomerulopathies (NPG group) were examined by percutaneous renal biopsy. As a control 12 biopsy specimens of the kidneys removed because of trauma were used. In the PG group the mean values of the glomerular immunoeexpression of NF- κ B were significantly increased as compared to both NPG patients and controls. In the PG but not in the MPG group glomerular immunoeexpression of NF- κ B was significantly positively correlated with the degree of proteinuria. Moreover, in the PG group glomerular immunoeexpression of NF- κ B was positively correlated with the mesangial cells, mesangial area and glomerular monocytes/macrophages. In conclusion, our results strongly suggest a role of NF- κ B in glomerular injury in proteinuric patients with proliferative glomerulopathies.

Key words: NF- κ B, proteinuric patients, glomerulonephritis.

Introduction

Transcription nuclear factor κ B (NF- κ B) is a pleiotropic transcription factor regulating the gene expression of several adhesion molecules, cytokines and chemotactic proteins involved in inflammation, immune response and cell proliferation [1-3]. It is a protein present as a homodimer or heterodimer of five members of the NF- κ B/Rel family. The most common dimer in many cell types is composed of subunits p50 and p65 [4-6]. This dimer is retained in an inactive form within the cytoplasm through non-covalent binding to inhibitory proteins called inhibitory κ B (I κ B). When activated by cytokines, mitogens, viruses or cell injury it moves to the nucleus, binds DNA and influences the

transcription of specific genes involved in inflammation such as cytokines and adhesion molecules; hence, it is present in a variety of chronic inflammatory disorders [7, 8]. Increasing data also suggest that NF- κ B plays a pivotal role in many glomerulopathies, especially immune-mediated [9, 10]. Immune complex formation and deposition in the kidney result in glomerular inflammation with recruitment of leukocytes, and the activation and proliferation of resident renal cells. However, cellular and molecular mechanisms underlying glomerular cell activation are not yet fully understood in human glomerulopathies [1]. Recent studies suggest that NF- κ B activation plays an essential role in the activation of mesangial cells and macrophages through the transcriptional induction of

inflammatory mediators of glomerular inflammation and injury [10, 11].

In view of the above, the aim of the present study was to determine glomerular immunoeexpression of NF- κ B (nuclear translocation of p65) in proteinuric patients with proliferative and non-proliferative glomerulopathies. Another purpose of this study was to examine the possible relationship between NF- κ B immunoeexpression and proteinuria, mesangial proliferation as well as glomerular inflammatory infiltrates.

Material and methods

Patients

Thirty-six patients with idiopathic proliferative glomerulopathies (PG group) who presented with proteinuria or nephrotic syndrome (mean age 39.5 ± 12.6) and 28 participants with non-proliferative glomerulopathies (NPG group) who presented with proteinuria or nephrotic syndrome (mean age 34.2 ± 14.7) were examined by percutaneous renal biopsy. The PG group included 14 cases with mesangiocapillary glomerulonephritis, 16 patients with IgA nephropathy and 6 with mesangial proliferative IgM glomerulopathy, whereas the NPG group consisted of 20 cases with membranous glomerulopathy and 8 with minimal changes. In all cases, diagnosis was based on characteristic findings by light microscopy (sections stained with hematoxylin and eosin, Masson-Trichrome, Jones' silver impregnation and periodic acid-Schiff followed by Alcian blue) as well as immunofluorescence (using antibodies against IgA, IgG, IgM, C3, C1q and light chains λ and κ). Moreover, in all patients electron microscopy was performed using standard protocols. Thickness of each section was controlled according to the method described by Weibel [12]. Most of our patients were middle-aged. Male predominance was noticeable in both PG and NPG groups. At the time of renal biopsy in the PG group 12 participants had nephrotic syndrome and 24 had proteinuria, whereas in the NPG group nephrotic syndrome was noted in 20 patients and proteinuria in 8. Hematuria accompanied proteinuria in 23 PG and 11 NPG cases. Clinical renal impairment (serum creatinine greater than 1.5 mg/dl) was noted in 6 PG and 2 NPG patients. Elevated blood pressure was found in 11 PG and 2 NPG individuals. As a control 12 biopsy specimens of the kidneys removed because of trauma were used (the male to female ratio was 8:4, the mean age was 39.1 ± 8.1). None of the persons from whom renal tissue originated were known to have had previous or current renal disease. Before the quantitative examinations were carried out, all control specimens were histologically examined by a nephropathologist and found to be normal renal tissue.

Immunohistochemistry

Paraffin sections were mounted onto superfrost slides, deparaffinized, then treated in a microwave oven in a solution of citrate buffer, pH 6.0 for 20 min and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 min, and then sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, Denmark) and incubated with: polyclonal rabbit-anti-human NF- κ B p65 (Immuno-Biological Laboratories Co., Ltd., dilution 5 μ g/ml), monoclonal mouse anti-human CD68 antibody (Clone KP-1, DakoCytomation, Denmark, dilution 1 : 100), monoclonal mouse anti-human CD3 T cell antibody (Clone PC3/188A, DakoCytomation, Denmark, dilution 1 : 50) and monoclonal mouse anti-human CD20 B cell antibody (Clone L-26, DakoCytomation, Denmark, dilution 1 : 200). Afterwards LSAB+/HRP Universal kit (DakoCytomation, Denmark) prepared according to the instructions of the manufacturer was used. Visualization was performed by incubating the sections in a solution of 0.5 mg 3,3'-diaminobenzidine (DakoCytomation, Denmark), per ml Tris-HCl buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10 min. After washing, the sections were counter-stained with hematoxylin and coverslipped. For each antibody and for each sample a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

Morphometry

Histological morphometry was performed by means of an image analysis system consisting of a PC equipped with a Pentagram graphical tablet, Indeo Fast card (frame grabber, true-color, real-time), produced by Indeo (Taiwan), and Panasonic color TV camera (Japan) coupled to a Carl Zeiss microscope (Germany). This system was programmed (MultiScan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects (semiautomatic function) and the surface area of a structure using a stereological net (with regulated number of points). The colored microscopic images were saved serially in the memory of the computer, and then quantitative examinations were carried out.

In all available glomeruli the immunoeexpression of NF- κ B, glomerular monocytes/macrophages, T lymphocytes and B lymphocytes were determined by counting p65+, CD68+, CD3 and CD20+ cells (semiautomatic function). The same method was used for counting glomerular mesangial cells (in PAS-Alcian blue staining). The results were expressed as a mean number of immunopositive cells or mesangial cells per glomerular cross-section.

Mesangial area per cent of total glomerular area (in PAS-Alcian blue staining) was measured using the point

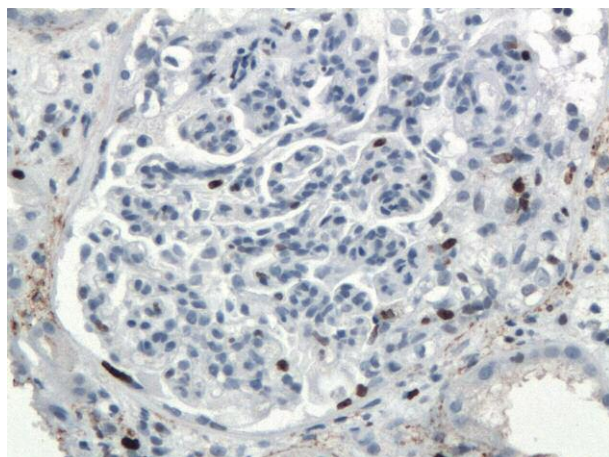


Fig. 1. PG group (mesangiocapillary glomerulonephritis). The immunorexpression of NF-κB in glomerular mesangial and epithelial cells. Magnification 400×

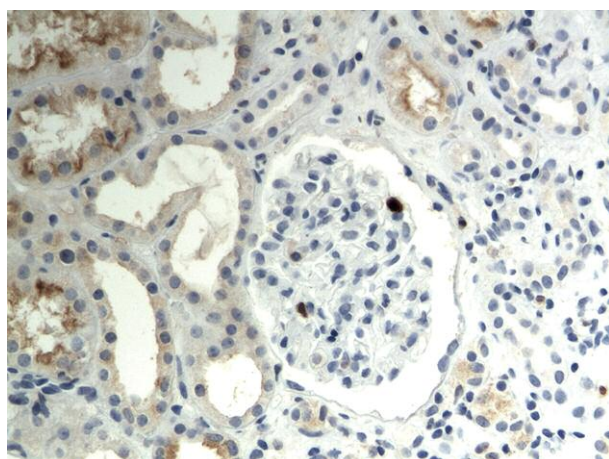


Fig. 2. NPG group (minimal change disease). The immunorexpression of NF-κB in some mesangial cells. Magnification 200×

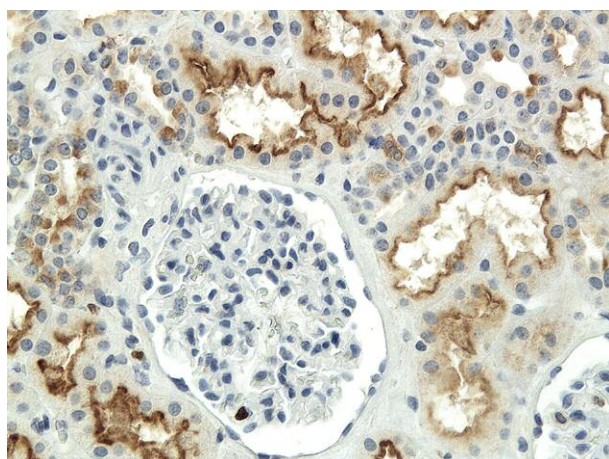


Fig. 3. Control group. Only one immunopositive cell within the glomerulus is seen. Magnification 200×

counting method, which is an adaptation of the principles of Weibel [12]. The point spacing was 16 μm. The total number of the points of a net was 169, and total area was 36864 sq. μm. The percentage of mesan-

gial area was an expression of the number of points overlying this structure as a percentage of the total points counted.

Statistical methods

Differences between groups were tested using unpaired Student's t-test preceded by evaluation of normality and Levene's test. The Mann-Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearman's method. Results were considered statistically significant if $p < 0.05$.

Results

Clinical features of the patients at the time of biopsy are given in Table I. The glomerular immunorexpression of NF-κB (mainly in mesangial cells but also in some endothelial and epithelial cells) in both PG and NPG groups with proteinuria was exclusively nuclear (Fig. 1 and Fig. 2.). In controls nuclear glomerular immunorexpression of NF-κB was very weak (Fig. 3). Nuclear immunorexpression of NF-κB was also seen in tubular epithelial cells and interstitial infiltrates, but for the present study it was not taken into consideration. A morphometric comparison of glomerular immunorexpression of NF-κB, mesangial parameters and glomerular inflammatory cells appears in Table II. In the PG group the mean values of the glomerular immunorexpression of NF-κB, mesangial cells, mesangial area and interstitial infiltrates (CD68+, CD3+ and CD20+ cells) were significantly increased as compared to both NPG patients and controls. A comparison of NPG patients and controls revealed that only the immunorexpression of NF-κB was significantly increased in the NPG group. The mesangial cells, mesangial area and glomerular monocytes/macrophages as well as T and B lymphocytes did not differ significantly in these groups. In the PG but not in the MPG group glomerular immunorexpression of NF-κB was significantly positively correlated with the degree of proteinuria. Moreover, in the PG group glomerular immunorexpression of NF-κB was positively correlated with the mesangial cells, mesangial area and glomerular monocytes/macrophages. In controls all correlations were weak and not significant (Table III).

Discussion

NF-κB regulates the gene expression of several cytokines and matrix proteins that are involved in inflammation [13, 14]. In the present study we revealed that glomerular immunorexpression of NF-κB was significantly increased in PG proteinuric patients as compared to the NPG group and controls. NF-κB activation has been observed in several experimental and human glomerular diseases [1, 15, 16]. Sakurai *et al.* noted increased activation of NF-κB in rat glomeruli shortly

Table I. Clinical and laboratory findings at the time of biopsy in cases with proteinuric proliferative and non-proliferative glomerulopathies

NUMBER OF CASES	GENDER (M/F)	MICRO-HEMA-TURIA	GROSS HEMA-TURIA	PROTEINURIA 1-2 G/24 H	PROTEINURIA 2-3.5 G/24 H	NEPHROTIC SYNDROME	RENAL FUNCTION IMPAIRMENT*	HYPERTENSION (> 90/160)
proliferative glomerulopathies (n = 36)	22/14	12	11	10	14	12	6	11
non-proliferative glomerulopathies (n = 28)	16/12	6	5	3	5	20	2	2

*serum creatinine > 1.5 mg%

Table II. Morphometric data of glomerular immunorexpression of NF-κB, mesangial parameters and glomerular inflammatory cells in proteinuric cases with proliferative and non-proliferative glomerulopathies as well as in controls

	PROLIFERATIVE GLOMERULOPATHIES (N = 36)	NON-PROLIFERATIVE GLOMERULOPATHIES (N = 28)	CONTROLS (N = 12)	P VALUE
NF-κB immunopositive cells/glomerular cross-section	2.3 ± 2.6	0.88 ± 0.7	0.27 ± 0.1	< 0.007 ^a , < 0.02 ^b , < 0.006 ^c
mesangial cells/glomerular cross-section	26.2 ± 11.3	17.4 ± 7.1	15.2 ± 6.3	< 0.001 ^a , < 0.003 ^b , = 0.35 ^c
mesangium (% of total glomerular area)	12.6 ± 3.8	9.2 ± 1.4	8.7 ± 1.1	< 0.001 ^a , < 0.002 ^b , = 0.27 ^c
CD68+ cells/glomerular cross-section	2.43 ± 1.7	1.28 ± 0.6	0.98 ± 0.4	< 0.002 ^a , < 0.006 ^b , = 0.12 ^c
CD3+ cells/glomerular cross-section	1.08 ± 0.65	0.32 ± 0.43	0.21 ± 0.13	< 0.001 ^a , < 0.001 ^b , = 0.39 ^c
CD20+ cells/glomerular cross-section	1.22 ± 0.71	0.15 ± 0.11	0.11 ± 0.10	< 0.001 ^a , < 0.001 ^b , = 0.28 ^c

Data are expressed as mean ± standard deviation

^a between proliferative and non-proliferative glomerulopathies^b between proliferative glomerulopathies and controls^c between non-proliferative glomerulopathies and controls**Table III.** Spearman rank order correlations between glomerular immunorexpression of NF-κB and proteinuria, mesangial cells, mesangial area as well as glomerular infiltrates

PAIR OF VARIABLES	PROLIFERATIVE GLOMERULOPATHIES (N = 36)	NON-PROLIFERATIVE GLOMERULOPATHIES (N = 28)	CONTROLS (N = 12)
glomerular immunorexpression of NF-κB and proteinuria	r = 0.51, p < 0.002	r = 0.355, p = 0.066 (NS)	–
glomerular immunorexpression of NF-κB and mesangial cells	r = -0.38, p < 0.03	r = 0.23, p = 0.24 (NS)	r = 0.34, p = 0.29 (NS)
glomerular immunorexpression of NF-κB and mesangial area	r = -0.46, p < 0.005	r = 0.23, p = 0.24 (NS)	r = 0.17, p = 0.59 (NS)
glomerular immunorexpression of NF-κB and glomerular CD68+ cells	r = -0.58, p < 0.001	r = 0.3232, p = 0.099 (NS)	r = 0.41, p = 0.18 (NS)
glomerular immunorexpression of NF-κB and glomerular CD3+ cells	r = -0.29, p = 0.08 (NS)	r = 0.16, p = 0.41 (NS)	r = 0.09, p = 0.78 (NS)
glomerular immunorexpression of NF-κB and glomerular CD20+ cells	r = 0.18, p = 0.29 (NS)	r = 0.19, p = 0.33 (NS)	r = 0.06, p = 0.85 (NS)

after nephrotoxic serum administration and the development of proteinuria. The effect peaked at days 3 to 5 and lasted about two weeks [17]. Our data support this observation as we found a significant positive correlation between glomerular immunoeexpression of NF-κB and proteinuria. Of note, this correlation in proteinuric NPG patients was also positive but not significant. Contrary to us, Zheng *et al.* showed a positive correlation of glomerular NF-κB also in the non-proliferative group. However, in this study glomerular NF-κB immunoeexpression was confined only to podocytes [1]. Our observations may suggest different mechanisms of proteinuria in proliferative and non-proliferative glomerulopathies. Although in our study, as may be expected, mesangial parameters and glomerular inflammatory infiltrates were significantly greater in the PG group in comparison to NPG patients and controls, the correlative study gave an interesting insight into the role of NF-κB in glomerular proliferative changes. In proliferative glomerulopathies we observed significant positive correlations between glomerular immunoeexpression of NF-κB and both mesangial cells and mesangial area, whereas these correlations were in NPG patients and controls weak and not significant. Similarly, Zheng *et al.* found a positive significant correlation between glomerular NF-κB activation and activity index in patients with lupus nephritis [1], whereas Peng *et al.* revealed the NF-κB inflammatory signaling pathway in rat glomerular mesangial cells cultured under high glucose [18]. In IgA nephropathy glomerular NF-κB expression correlated with progression of tissue injury [15]. It was also revealed that increased NF-κB activation in mesangial cells correlates with increased expression of a variety of inflammatory genes such as interleukins IL-1β, IL-6 and IL-8, tumor necrosis factor α (TNF-α) monocyte chemoattractant protein-1 (MCP-1), interferon invasive protein-10 (IP-10), and inducible nitric oxide synthase [2, 10, 19-23]. In addition to the pro-inflammatory actions of NF-κB, some data, similarly as our study, support a role for NF-κB in the control of mesangial cell proliferation [2, 24-28].

Another important finding in the present study was that in PG but not in NPG and control patients a significant positive correlation existed between glomerular immunoeexpression of NF-κB and glomerular CD 68+ cells. Similarly, Zheng *et al.* found that the degree of glomerular macrophage infiltration was positively correlated with the number of mesangial and endothelial cells positive for NF-κB [1]. Moreover, Hisada *et al.* observed in cultured cells close interaction between mesangial cells and monocytes/macrophages through NF-κB activation [29]. These observations suggest that overexpression of NF-κB in glomerular cells may play a pathogenic role in glomerular inflammation and injury in proliferative glomerulopathies.

On the other hand, we found that correlations between glomerular immunoeexpression of NF-κB and glomerular T lymphocytes and B lymphocytes were weak and not significant in all groups. These results may suggest that in glomeruli CD3+ and CD20+ cells are not involved in the NF-κB signaling pathway in human glomerulopathies.

In conclusion, our results strongly suggest a role of NF-κB in glomerular injury in proteinuric patients with proliferative glomerulopathies.

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References

- Zheng L, Sinniah R, Hsu SI. In situ glomerular expression of activated NF-kappaB in human lupus nephritis and other non-proliferative proteinuric glomerulopathy. *Virchows Arch* 2006; 448: 172-183.
- Guijarro C, Egidio J. Transcription factor-kappa B (NF-kappa B) and renal disease. *Kidney Int* 2001; 59: 415-424.
- Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2002; 2: 725-734.
- Baldwin AS. The NF-κB and IκB proteins: New discoveries and insights. *Annu Rev Immunol* 1996; 14: 649-681.
- Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998; 16: 225-260.
- Grilli M, Chiu JJ, Lenardo MJ. NF-κB and Rel: participants in a multiform transcriptional regulatory system. *Int Rev Cytol* 1993; 143: 1-62.
- Auwardt RB, Mudge SJ, Power DA. Transcription factor NF-κB in glomerulonephritis. *Nephrology* 2000; 5: 71-82.
- Barnes PJ, Karin M. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-1071.
- Morrissey J, Klahr S. Transcription factor NF-kappaB regulation of renal fibrosis during ureteral obstruction. *Semin Nephrol* 1998; 18: 603-611.
- Massy ZA, Guijarro C, O'Donnell MP, et al. The central role of nuclear factor-κB in mesangial cell activation. *Kidney Int Suppl* 1999; 71: S76-79.
- Kitamura M. Adoptive transfer of nuclear factor-kappaB-inactive macrophages to the glomerulus. *Kidney Int* 2000; 57: 709-16.
- Weibel ER. Point Counting Methods. In: Weibel ER. *Stereological Methods*. vol. 1. Academic Press, London, New York, Toronto, Sydney, San Francisco 1979; 101-159.
- Barnes PJ, Karin M. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066-1071.
- Mezzano SA, Barría M, Droguett MA et al. Tubular NF-kappaB and AP-1 activation in human proteinuric renal disease. *Kidney Int* 2001; 60: 1366-1377.
- Ashizawa M, Miyazaki M, Abe K, et al. Detection of nuclear factor-kappaB in IgA nephropathy using Southwestern histochemistry. *Am J Kidney Dis* 2003; 42: 76-86.
- Mudge SJ, Paizis K, Auwardt RB, et al. Activation of nuclear factor-kappa B by podocytes in the autologous phase of passive Heymann nephritis. *Kidney Int* 2001; 59: 923-931.
- Sakurai H, Hisada Y, Ueno M, et al. Activation of transcription factor NF-kappa B in experimental glomerulonephritis in rats. *Biochim Biophys Acta* 1996; 1316: 132-138.

18. Xie X, Peng J, Huang K, et al. Polydatin ameliorates experimental diabetes-induced fibronectin through inhibiting the activation of NF- κ B signaling pathway in rat glomerular mesangial cells. *Mol Cell Endocrinol* 2012; 362: 183-189.
19. Wilmer WA, Tan LC, Dickerson JA, et al. Interleukin-1 β induction of mitogen-activated protein kinases in human mesangial cells. Role of oxidation. *J Biol Chem* 1997; 272: 10877-10881.
20. Guijarro C, Kim Y, Kasiske BL, et al. Central role of the transcription factor nuclear factor-kappa B in mesangial cell production of chemokines. *Contrib Nephrol* 1997; 120: 210-218.
21. Khachigian LM, Collins T, Fries JW. Nuclear factor-kappa B mediates induction of vascular cell adhesion molecule-1 in glomerular mesangial cells. *Biochem Biophys Res Commun* 1995; 206: 462-467.
22. Auwardt RB, Mudge SJ, Chen CG, et al. Regulation of nuclear factor kappaB by corticosteroids in rat mesangial cells. *J Am Soc Nephrol* 1998; 9: 1620-1628.
23. Ruiz-Ortega M, Bustos C, Hernández-Presa MA. Angiotensin II participates in mononuclear cell recruitment in experimental immune complex nephritis through nuclear factor-kappa B activation and monocyte chemoattractant protein-1 synthesis. *J Immunol.* 1998; 161: 430-439.
24. Guijarro C, Kim Y, Schoonover CM, et al. Lovastatin inhibits lipopolysaccharide-induced NF-kappaB activation in human mesangial cells. *Nephrol Dial Transplant* 1996; 11: 990-996.
25. Ortego M, Bustos C, Hernández-Presa MA et al. Atorvastatin reduces NF-kappaB activation and chemokine expression in vascular smooth muscle cells and mononuclear cells. *Atherosclerosis* 1999; 147: 253-261.
26. Massy ZA, Guijarro C, O'Donnell MP, et al. Regulation of mesangial cell proliferation by the mevalonate pathway. *Contrib Nephrol* 1997; 120: 191-196.
27. O'Donnell MP, Kasiske BL, Kim Y, et al. Lovastatin inhibits proliferation of rat mesangial cells. *J Clin Invest* 1993; 91: 83-87.
28. Sugiyama H, Savill JS, Kitamura M. Selective sensitization to tumor necrosis factor-alpha-induced apoptosis by blockade of NF-kappaB in primary glomerular mesangial cells. *J Biol Chem* 1999; 274: 19532-19537.
29. Hisada Y, Sakurai H, Sugaya T. Cell to cell interaction between mesangial cells and macrophages induces the expression of monocyte chemoattractant protein-1 through nuclear factor-kappaB activation. *Biochem Biophys Res Commun* 2000; 269: 309-316.

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