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The Glomerulosclerosis of Aging in Females

Contribution of the Proinflammatory Mesangial Cell Phenotype to Macrophage Infiltration

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Age-associated renal changes may be an important cause of renal failure. We recently found that aged female B6 mice developed progressive glomerular lesions. This was associated with macrophage infiltration, a frequent finding in glomerulosclerosis. We used these mice as a model for studying the mechanisms of glomerular aging. We compared the gene expression profile of intact glomeruli from late postmenopausal (28-month-old) mice to that of intact glomeruli from premenopausal (5-month-old) mice. We found that inflammation-related genes, especially those expressed by activated macrophages, were up-regulated in the glomeruli of 28-month-old mice, a result correlating with the histological observation of glomerular macrophage infiltration. The mechanism for macrophage recruitment could have been stable phenotypic changes in mesangial cells because we found that mesangial cells isolated from 28-month-old mice expressed higher levels of RANTES and VCAM-1 than cells from 5-month-old mice. The elevated serum tumor necrosis factor (TNF)- α levels present in aged mice may contribute to increased RANTES and VCAM-1 expression in mesangial cells. Furthermore, cells from 28-month-old mice were more sensitive to TNF- α -induced RANTES and VCAM-1 up-regulation. The effect of TNF- α on RANTES expression was mediated by TNF receptor 1. Interestingly, mesangial cells isolated from 28-month-old mice had

increased nuclear factor- κ B transcriptional activity. Inhibition of nuclear factor- κ B activity decreased baseline as well as TNF- α -induced RANTES and VCAM-1 expression in mesangial cells isolated from 28-month-old mice. Thus, phenotypic changes in mesangial cells may predispose them to inflammatory stimuli, such as TNF- α , which would contribute to glomerular macrophage infiltration and inflammatory lesions in aging. (*Am J Pathol* 2004, 165:1789–1798)

Three percent of the civilian, noninstitutionalized United States population (age, ≥ 17 years) has an elevated serum creatinine, according to the third National Health and Nutrition Survey.¹ The incidence increased with age. At the age of 65 or older, 11% of individuals had decreased renal function, even when corrected for obvious causes of renal diseases such as hypertension and diabetes mellitus. Thus, age-associated renal disease presents an important health problem.² Glomerulosclerosis is the most common pathological finding in those with chronic renal failure as a part of the aging process.³ Genetic susceptibility, a reduction in nephron number, an imbalance of redox status, the loss of sex hormones, as well as the accumulation of advanced glycation end products have all been implicated as important factors in the pathogenesis of aging-associated glomerulosclerosis.^{3–8} We found that C57BL/6 (B6) mice were resistant to glomerulosclerosis before menopause, but developed progressive glomerular lesions after menopause.⁹ Glomerular lesions were characterized by hypertrophy, vascular pole sclerosis, and mesangial cell proliferation dur-

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ing the early menopausal period (18 to 20 months of age) and moderate but more diffuse mesangial sclerosis at 22 to 24 months of age. In the late menopausal period (28 to 32 months of age), glomerulosclerosis was severe and was accompanied by macrophage infiltration. Because the glomerular lesions occurred in the absence of diabetes and hypertension, these mice provide a model for studying the molecular mechanisms of glomerulosclerosis in aging female mice.

Although genes in the insulin and insulin-like growth factor-1 signaling pathway have been shown to be involved in aging, the abnormalities in gene expression in aging have been shown to be complex.^{10,11} Microarray provides a high-throughput method for obtaining information on expression profiles of many genes and allows their comparison between multiple organs.¹¹ Altered gene expression in immune reactions, stress responses, and metabolic pathways have been found in the liver, skeletal muscle, heart, and brain of aged C57 B6 mice using microarray analysis.^{12–15} Herein we found increased glomerular expression of proinflammatory genes, especially genes expressed by activated macrophages, in 28-month-old female B6 mice. This expression profile correlated with the development of inflammatory lesions with prominent macrophage infiltration in the glomeruli of 28-month-old mice that we had previously reported.⁹ The basis for glomerular macrophage infiltration remained unclear. Mesangial cells have been suggested to play a role in macrophage infiltration and inflammatory lesions in various forms of glomerulonephritis.^{16–18} We speculated that mesangial cell phenotypic changes may contribute to glomerular macrophage infiltration in aging.

We found that the phenotypic changes of mesangial cells in aging, including increased nuclear factor (NF)- κ B activity, responses to tumor necrosis factor (TNF)- α , and higher levels of RANTES (regulated on activation, normal T cell expressed and secreted) and VCAM-1 (vascular cell adhesion molecule-1) expression, may play a role in the glomerular inflammatory lesions of aging.

Materials and Methods

Mice

Young and old female C57BL6 (B6) mice were obtained from the National Institute on Aging, National Institutes of Health, Bethesda, MD. Female B6 mice have irregular, lengthened estrous cycles between 10 to 14 months of age and cycles usually cease at 18 to 20 months of age.¹⁹ The normal life span of female B6 mice is ~32 to 34 months. Because we found that 28-month-old female B6 mice developed diffuse and severe glomerulosclerosis, mice of this age were used to represent the aged group. Young controls were 5-month-old female B6 mice, which had normal renal histology. Three-month-old male TNF receptor 1 (TNFR1) or TNFR2 knockout mice on the B6 background were originally obtained from Dr. Peschon (Immunex Corp., Seattle, WA).^{20,21}

Glomerular Microdissection and RNA Isolation

Glomeruli were microdissected from 5- and 28-month-old female B6 mice as previously described.⁹ For microarray analysis, 500 glomeruli from each mouse were collected in RNA later solution (Qiagen Inc., Valencia, CA). One thousand five hundred glomeruli from three mice were pooled and total RNA was isolated using the RNeasy kit based on the manufacturer's protocol (Qiagen Inc.). The quality of RNA was determined by measuring the A260/280 ratio with a spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). Because of the limited amount of RNA obtained from microdissected glomeruli, we used RNA from remnant kidney tissue after microdissection to run agarose gels to determine RNA quality. We postulated that the quality of remnant kidney tissue RNA and that from microdissected glomeruli would be similar because the length of time *ex vivo* and the RNA isolation techniques were identical.

Microarray

Microarray analyses were performed using the Affymetrix GeneChip System in the Gene Expression Profiling Core, The Center for Translational Respiratory Medicine, Johns Hopkins University School of Medicine.^{22,23} A protocol that performs two cycles of amplification was used (GeneChip Eukaryotic small sample target labeling technical note, 2002; Affymetrix, Inc., Santa Clara, CA). Briefly, in the first cycle of amplification, total RNA (100 ng) from each sample was converted into double-stranded cDNA using the SuperScript Choice system (Invitrogen Corp., Carlsbad, CA) with an oligo-dT primer containing T7 RNA polymerase promoter. The double-stranded cDNA was then used for *in vitro* transcription to synthesize cRNA using MEGAscript T7 Kit (Ambion, Austin, TX). In the second cycle of amplification, cRNA (250 ng) was used for the first strand cDNA synthesis with random primers. Second strand cDNA was synthesized with T7-(dT)₂₄ primer. *In vitro* transcription for cRNA amplification and biotin labeling were done using the ENZO BioArray RNA transcript labeling kit (Affymetrix, Inc.). Biotin-labeled cRNA was fragmented and hybridized (16 hours) to the Affymetrix murine genome U74Av2. The hybridized biotinylated cRNA was stained with phycoerythrin-streptavidin and was quantitated by scanning the fluorescent image (Agilent GeneArray scanner; Agilent Technologies, Palo Alto, CA). The quality of RNA and cRNA had been monitored on an Agilent 2100 bioanalyzer. External standards were included in each hybridization to control for hybridization efficiency, to test for sensitivity, and to assist in the comparisons between data sets. After positive analysis of the test chip, the same hybridization mixture was added to the expression chip and processed as described. Analysis of the scanned data was performed using Affymetrix software (MAS 5.0). Every chip was globally normalized to a target intensity of 150. Other parameters such as noise, background, scaling factor, and 3' to 5' ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin were within the normal

limits of Affymetrix standard. Two glomerular RNA samples (each contained a pool of RNA of glomeruli from three mice) from 5- or 28-month-old mice were examined. After pair-wise comparison among four sets data from the samples, gene expression levels that had a difference of twofold or greater between the glomeruli of 5- and 28-month-old mice were arbitrarily selected. This selection was tested by performing an unsupervised hierarchical computer clustering analysis of gene expression in glomeruli from 5- and 28-month-old mice using software Cluster (version 2.20) and Treeview (version 1.60; <http://rana.lbl.gov/EisenSoftware.htm>).²⁴

Validation of Gene Expression Data

To confirm the results of microarray analysis, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) on glomerular RNA isolated from 5- or 28-month-old mice. The primers used for PCR were: RANTES (forward, 5'-CATCCTCACTGCAGCCGCC; reverse, 5'-CCAAGCTGGCTAGGACTAGAG, 321 bp), VCAM-1 (forward, 5'-CAAGGGTGACCAGCTCATGAA; reverse, 5'-TGTGCAGCCACCTGAGATC, 518 bp), macrophage metalloelastase (MMP-12) (forward, 5'-GATGGCAAAGGTGGTACACT; reverse, 5'-GGT GACACGACGGAA-CAGGG, 852 bp), CC chemokine receptor 5 (CCR5) (forward, 5'-AATTCTTTGGACTGAATAACTGCA; reverse, 5'-GTGGATCGGGTATAGACTGAGCTT, 236 bp), MCP-1 (forward, 5'-CTCACCTGCTGCTACTCATTC; reverse, 5'-GCTTGAGGTGGTTGTGGAAAA, 319 bp), and ICAM-1 (forward, 5'-TCGGAGGATCACAAACGAAGC; reverse, 5'-AACATAAGAGGCTGCCATCACG, 432 bp). The amplification consisted of denaturation for 30 seconds at 94°C, a 30-second annealing at 60°C, and a 45-second extension at 72°C. The optimal number of PCR cycles was 32 for VCAM-1; 35 for ICAM-1, RANTES, and MCP-1; 38 for CCR5; and 40 for MMP-12. GAPDH mRNA was determined by standard and competitive PCR as previously described.⁹

Mesangial Cell Culture

Mesangial cells were isolated from 5- and 28-month-old female B6 mice and characterized as previously described.⁹ At least two cell lines from each age group were obtained and found to be indistinguishable in the assays we used. Cells were grown in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 20% fetal bovine serum and 100 μ /ml each of streptomycin and penicillin. Cells at passage between 8 to 20 were used for the experiments. To test the effects of TNF- α on RANTES and VCAM-1 mRNA expression, cells were seeded at the density of 3×10^5 /well in a six-well plate with medium containing 20% fetal bovine serum for 24 hours. Then the medium was switched to DMEM/F12 medium containing 0.1% fetal bovine serum. The reconstituted DMEM containing recombinant human TNF- α (Genzyme, Cambridge, MA) at a concentration of 5, 10, or 20 ng/ml was added to the cells 24 hours later. Total RNA was collected from the cells 6 hours after TNF- α

treatment. mRNA levels of RANTES and VCAM-1 in mesangial cells were quantitated by competitive PCR. The RANTES mutant was created by deletion of 132 bp using the primers (5'-TGCCTCACCATATGGAGTGTGTGCAACCCAG; 3'-CTGGGTTGGCACACTCCATATGGT-GAGGCA). The VCAM-1 mutant was created by deletion of 119 bp using the primers (5'-TGGTGCTGTGACAGGAAGACTCTGGAGTCT; 3'-AGACTCCAGAGTCTTCCTGTCACAGCACCA). Competitive PCR was performed by adding decreasing amounts of mutant to sample tubes as previously described.²⁵ GAPDH mRNA levels were measured by competitive PCR in the same sample. The expression of TNFR1 and TNFR2 mRNA in mesangial cells isolated from 5- and 28-month-old mice was examined by RT-PCR. TNFR1 primers were: forward, 5'-CCGAAGTCTACTCCATCATTGTGTA and reverse, 5'-ACGCCATCCACCACAGCATACA. TNFR2 primers were: forward, 5'-ATGCCATGCTCACCGATTCCAC and reverse, 5'-AACCCGTCTCCTTCCCACAACA. To examine the role of two cell surface receptors TNFR1 and TNFR2 in TNF- α -mediated RANTES and VCAM-1 expression in mesangial cells, we isolated and propagated mesangial cells from TNFR1 and TNFR2 knockout male mice. The levels of RANTES and VCAM-1 mRNA expression in TNFR1- or TNFR2-deficient mesangial cells in the presence or absence of TNF- α stimulation were determined as described above.

Transfection and NF- κ B Reporter Gene Assay

Transient transfection was performed in mesangial cells from 5- and 28-month-old mice using Transfast according to the manufacturer's protocol (Promega, Madison, WI).²⁶ Cells were transfected with a NF- κ B reporter in the presence or absence of a cDNA expression vector containing an inhibitor of NF- κ B α (I κ B α), a cDNA expression vector containing a I κ B kinase (IKK), or a cDNA expression vector containing a dominant-negative IKK (IKKDN). A β -galactosidase cDNA expression vector was co-transfected with the NF- κ B reporter to serve as an internal control for transfection efficiency. The effect of TNF- α on mesangial cell NF- κ B transcriptional responses was determined by adding 5 to 10 ng/ml of recombinant human TNF- α to NF- κ B reporter-transfected cells. Cells were harvested 24 hours after transfection. Luciferase and β -galactosidase activity were measured using substrate assays as previously described.²⁶ The same amount of DNA (500 ng/well of 24-well plates) was used for each transfection throughout the experiments.

Inhibition of NF- κ B in Mesangial Cells

Subconfluent mesangial cells isolated from 28-month-old mice were incubated in six-well plates in medium containing 0.1% fetal calf serum for 24 hours. Pyrrolidinedithiocarbamate (PDTC, 0.075 μ mol/L), or dimethyl sulfoxide was added to the cells 2 hours before treatment with TNF- α (5 ng/ml). Total RNA was collected 6 hours after TNF- α incubation. We also transfected mesangial cells from 28-month-old mice with an IKKDN expression vector

Table 1. Up-Regulated Genes in Intact Glomeruli of 28-Month-Old Female B6 Mice

GenBank code	Gene	Fold difference (28 months versus 5 months)
Immune reaction		
X67210	Rearranged immunoglobulin gamma 2b heavy chain	7.1
M82831	Macrophage metalloelastase	5.1
AF065947	Small inducible cytokine A5 (ScyA5)	4.8
M21050	Lysozyme M gene	4.2
M22531	Complement component 1 q, B chain	4.2
AF030636	CXC chemokine (angie2) mRNA, complete cds	3.7
X16834	mRNA for Mac-2 antigen	3.5
X68273	CD68 antigen	3.2
X52643	Histocompatibility 2, class II antigen A, alpha	3.2
D37837	65-kd macrophage cytosolic protein	3.0
M31039	Complement receptor 3 beta subunit MAC-1/mRNA	3.0
M84487	Vascular cell adhesion molecule 1	2.5
K02782	Complement component C3 mRNA, alpha and beta subunits	2.4
M32489	Interferon consensus sequence binding protein	2.2
Extracellular matrix		
D00613	Matrix gamma-carboxyglutamate protein	2.6
M15832	Procollagen, type IV, alpha 1	2.2
U03419	alpha I type I procollagen mRNA	2.0
Others		
X81580	Insulin-like growth factor binding protein 2	4.0
X53929	Decorin	3.2
X78445	Cyp1-b1 mRNA for cytochrome P450	2.4
L19332	Transforming growth factor, beta induced, 68 kd	2.4
X66449	Calcyclin	2.2
U49430	Ceruloplasmin	2.2
X004480	Insulin-like growth factor 1	2.2
AF05618	Insulin-like growth factor 1 receptor mRNA	2.0

or an empty pCR3 vector to examine the effect of NF- κ B inhibition on TNF- α mediated RANTES and VCAM-1 expression. TNF- α or the same volume of DMEM media was added to the cells 16 hours after transfection. Cells were collected 6 hours later. RANTES and VCAM-1 mRNA levels were determined by competitive PCR.

Serum TNF- α Levels

Sera were obtained from 3- to 9-month-old ($n = 9$) and 22- to 28-month-old ($n = 11$) female B6 mice. Serum TNF- α levels were measured by enzyme-linked immunosorbent assay using a kit from Biosource International, Inc. (Camarillo, CA). Serum or mouse TNF- α (19.5 to 625 pg/ml) was added to plates that had been precoated with polyclonal anti-mouse TNF- α antibody and incubated with a biotinylated second antibody at room temperature for 90 minutes. Antigen-antibody reaction was revealed with a streptavidin-peroxidase substrate. Serum TNF- α levels were calculated from a standard curve.

Statistical Methods

Except for microarray analysis data, values were expressed as means $\pm$ 1 SD. One-way analysis of variance or Student's *t*-test was used. $P < 0.05$ was considered to be statistically significant.

Results

Gene Expression in Intact, Isolated Glomeruli from 28-Month-Old Mice

A total of 12,473 oligonucleotide probes were used for the microarray analyses, 4423 were hybridized to glomerular cRNA from 28-month-old mice (35.5%) and 5020 were hybridized to glomerular cRNA from 5-month-old mice (40.2%). We selected only probes that hybridized with both 5- and 28-month-old glomerular samples, recognizing that this may result in the loss of some important information. Twenty-five genes of known function were up-regulated in glomerular cRNA from 28-month-old mice compared to that from 5-month-old mice (Table 1). An increase in gene transcripts related to inflammatory responses was a prominent feature in the cRNA of glomeruli from 28-month-old mice. An unsupervised hierarchical computer clustering analysis validated this data.

Confirmation of Microarray Results by Standard RT-PCR

A 5.3-fold increase in RANTES, a 3.5-fold increase in VCAM-1, and a 3.3-fold increase in macrophage elastase (MMP-12) mRNA levels in glomeruli from 28-month-old mice were found using RT-PCR analysis (Figure 1). The levels of CCR5 mRNA expression were not different between glomeruli from 28- and 5-month-old mice. We pre-

Figure 1. Validation of genes found to be up-regulated by gene expression chip analysis in intact glomeruli of 28-month-old female B6 mice. Glomerular RNA were isolated from 5- and 28-month-old female B6 mice ($n = 6$ for each age group). Glomerular mRNA levels were measured by standard RT-PCR. **A:** Increased RANTES expression in glomeruli of 28-month-old B6 mice. RANTES mRNA levels were measured by RT-PCR (representative gel: 1, 2, 3 = glomerular samples of 5-month-old B6 mice; 4, 5, 6 = glomerular samples of 28-month-old B6 mice). Densitometric analysis shows increased RANTES mRNA levels in glomeruli of 28-month-old mice. **, $P < 0.01$ versus 5-month-old mice. **B:** Increased VCAM-1 expression in glomeruli of 28-month-old mice. Representative gel of PCR (1, 2, 3 = glomerular samples of 5-month-old mice; 4, 5, 6 = glomerular samples of 28-month-old mice). Densitometric analysis indicates increased VCAM-1 mRNA levels in glomeruli of 28-month-old mice. **, $P < 0.01$ versus 5-month-old mice. **C:** Increased MMP-12 expression in glomeruli of 28-month-old mice. Representative gel of PCR is shown (1, 2, 3 = glomerular samples of 5-month-old mice; 4, 5, 6 = glomerular samples of 28-month-old mice). Densitometric analysis indicates increased MMP-12 mRNA levels in glomeruli of 28-month-old mice. *, $P < 0.01$ versus 5-month-old mice. **D:** No change in CCR5 mRNA expression in glomeruli of 28-month-old mice.

viously found increased $\alpha 1$ type I and $\alpha 1$ type IV collagen mRNA expression in glomeruli from 28-month-old mice.⁹ There were no differences in MCP-1 or ICAM-1 mRNA levels between glomeruli from 5- and 28-month-old mice (data not shown).

Mesangial Cell RANTES, VCAM-1, TNFR1, and TNFR2 mRNA Levels

The levels of RANTES and VCAM-1 mRNA were higher at baseline in two lines of mesangial cells isolated from 28-month-old than in two other lines isolated from 5-month-old mice (2.1-fold and 2.3-fold, respectively) (Figure 2). There were no differences in TNFR1 and TNFR2 mRNA levels between mesangial cells from 5- and 28-month-old mice (data not shown). The expression of RANTES and VCAM-1 mRNA was up-regulated by TNF- α in mesangial cells from both 5- and 28-month-old mice, but there was a twofold increase in sensitivity in those from 28-month-old mice (Figure 3). TNF- α at a dose of 5 ng/ml stimulated maximal RANTES and VCAM-1 expression in mesangial cells from 28-month-old mice, whereas a TNF- α dose of 10 ng/ml was required to stimulate either RANTES or VCAM-1 expression in mesangial

cells from 5-month-old mice. No further increase in RANTES and VCAM-1 expression was found in either group of mesangial cells at a dose of 20 ng/ml of TNF- α . However, there was a difference in the maximal levels of RANTES expression between mesangial cells from the two age groups. Although responsive to TNF- α treatment, the poststimulation levels of RANTES mRNA in mesangial cells from 28-month-old mice remained nearly 50% higher than that in mesangial cells from 5-month-old mice. The levels of VCAM-1 after stimulation with 10 ng/ml of TNF- α were comparable between mesangial cells from 5- and 28-month-old mice.

RANTES and VCAM-1 mRNA Expression in Mesangial Cells Lacking TNFR1 or TNFR2

Mesangial cells lacking TNFR1 had much lower levels of RANTES and VCAM-1 expression than mesangial cells lacking TNFR2 (Figure 4). Furthermore, RANTES expression in mesangial cells lacking TNFR1 was not induced by TNF- α stimulation, even when TNF- α levels were increased to 20 ng/ml. However, TNF- α induced a 3.6-fold increase in VCAM-1 expression in TNFR1-deficient mesangial cells. This suggested that RANTES and VCAM-1

Figure 2. Increased RANTES and VCAM-1 expression in mesangial cells isolated from 28-month-old female B6 mice. RANTES and VCAM-1 mRNA levels were measured by competitive PCR using cDNAs from mesangial cells cultured from 5-month-old (5-month MC) and 28-month-old (28-month MC) female B6 mice. Two separate mesangial cell lines from 5-month-old or 28-month-old mice were examined. RANTES and VCAM-1 mRNA levels in mesangial cells were normalized to GAPDH mRNA levels (measured by competitive PCR). The ratio of RANTES or VCAM-1 to GAPDH mRNA in 5-month MC was arbitrarily defined as 100%. **A:** Representative competitive PCR, RANTES. cDNAs from 5-month MC and 28-month MC were amplified with decreasing amounts of a competing mutant (from **left** to **right**: 0.1 to 0.00313 attmol). **B:** RANTES mRNA levels were increased in mesangial cells isolated from 28-month-old mice. **, $P < 0.01$ versus 5-month MC. **C:** Representative competitive PCR, VCAM-1. cDNAs from 5-month MC and 28-month MC were amplified with decreasing amounts of a competing mutant (from **left** to **right**: 0.5 to 0.0156 attmol). **D:** VCAM-1 mRNA levels were increased in mesangial cells isolated from 28-month-old mice. **, $P < 0.01$ versus 5-month MC.

responses were independently regulated. The response to TNF- α in mesangial cells lacking TNFR2 was normal, namely, there was up-regulation of RANTES and VCAM-1.

Increased NF- κ B Transcriptional Activity in Mesangial Cells Isolated from 28-Month-Old Mice

Mesangial cells were transfected with a NF- κ B reporter vector in the presence or absence of TNF- α . Basal

NF- κ B transcriptional activity was present in mesangial cells from both 5- and 28-month-old mice (Figure 5). However, baseline activity of the NF- κ B reporter gene was 2.6-fold higher in mesangial cells from 28-month-old mice than in mesangial cells from 5-month-old mice. As expected, co-transfection with an I κ B α or an IKK dominant-negative expression vector significantly decreased NF- κ B reporter activity. Whereas, an IKK expression vector significantly increased NF- κ B reporter activity. TNF- α at a dose of 5 ng/ml increased

Figure 3. Increased sensitivity to TNF- α induced RANTES and VCAM-1 up-regulation in mesangial cells isolated from 28-month-old female B6 mice. Mesangial cells isolated from 5-month-old (5-month MC) and 28-month-old (28-month MC) B6 mice were treated with 5, 10, or 20 ng/ml of TNF- α (diluted with DMEM) for 6 hours. DMEM-treated cells were used as controls. Duplicate experiments were performed in two separate cell lines from 5-month-old or 28-month-old mice. Total RNA was collected from cells. RANTES and VCAM-1 mRNA levels were measured by competitive PCR and corrected to GAPDH mRNA levels. The ratio of RANTES or VCAM-1 to GAPDH mRNA in untreated 5-month MC was arbitrarily defined as 100%. **A:** TNF- α at a level of 5 ng/ml increased RANTES expression in 28-month MC but not in 5-month MC. TNF- α at a level of 10 ng/ml was required to induce up-regulation of RANTES expression in 5-month MC. A further increase in TNF- α did not induce increased expression of RANTES in either 5-month MC or 28-month MC ($P > 0.05$). Note that RANTES mRNA levels in 10 ng/ml TNF- α -treated 5-month MC were lower than in 5 ng/ml TNF- α -treated 28-month MC. ($P < 0.05$) **B:** TNF- α at a level of 5 ng/ml increased VCAM-1 expression in 28-month MC but not in 5-month MC. TNF- α at a level of 10 ng/ml was required to induce up-regulation of VCAM-1 expression in 5-month MC. A further increase in TNF- α levels did not induce increased expression of VCAM-1 in either 5-month MC or 28-month MC. Note that the mRNA levels of VCAM-1 were comparable between 10 to 20 ng/ml TNF- α -treated 5-month MC and 5 to 20 ng/ml TNF- α -treated 28-month MC.

Figure 4. The up-regulation of RANTES by TNF- α is abolished in mesangial cells lacking TNF- α receptor 1 (TNFR1). Mesangial cells were isolated from TNFR1 and from TNFR2 knockout mice. The baseline and TNF- α -stimulated expression of RANTES and VCAM-1 in mesangial cells isolated from TNFR1-deficient or TNFR2-deficient mice were measured by competitive RT-PCR. RANTES or VCAM-1 mRNA levels were normalized to GAPDH mRNA levels. The ratio of RANTES or VCAM-1 to GAPDH in mesangial cells lacking TNFR1 (R1) at baseline was arbitrarily defined as 100%. **A:** Representative competitive PCR, RANTES. cDNAs from 20 ng/ml TNF- α -treated mesangial cells lacking either TNFR1 (R1) or TNFR2 (R2) were amplified with decreasing amounts of a competing mutant (from **left** to **right**: 0.4 to 0 attmol). **B:** At baseline, RANTES mRNA levels were significantly higher in R2 than in R1. TNF- α induced up-regulation of RANTES expression in R2 but not in R1. **, $P < 0.01$ versus R1 at baseline. ##, $P < 0.01$ versus R2 at baseline. **C:** Representative competitive PCR, VCAM-1. cDNAs from 20 ng/ml TNF- α -treated R1 or R2 were amplified with decreasing amounts of a competing mutant (from **left** to **right**: 1 to 0 attmol). **D:** At baseline, VCAM-1 mRNA levels were significantly higher in R2 than in R1. TNF- α induced up-regulation of VCAM-1 expression in both R1 and R2. &&, $P < 0.01$ versus R1 at baseline; **, $P < 0.01$ versus R1 at baseline; #, $P < 0.05$ versus R2 at baseline.

NF- κ B transcriptional activity in mesangial cells from 28-month-old, but not in those from 5-month-old mice. However, TNF- α at a concentration of 10 ng/ml up-regulated NF- κ B transcriptional activity in mesangial cells from 5-month-old mice. Thus, NF- κ B activity was more sensitive to TNF- α stimulation in 28-month-old than in 5-month-old mice mesangial cells.

Inhibition of NF- κ B Decreased RANTES and VCAM-1 Expression in Mesangial Cells Isolated from 28-Month-Old Mice

Inhibition of NF- κ B activity in mesangial cells from 28-month-old mice by PDTC reduced RANTES and VCAM-1 expression to levels comparable to that in mesangial

Figure 5. Increased NF- κ B transcriptional activity in mesangial cells isolated from 28-month-old mice. Two separate mesangial cell lines were cultured from 5-month-old (5-month MC) or 28-month-old (28-month MC) female B6 mice. NF- κ B transcriptional activity was measured by transfecting cells with a luciferase NF- κ B reporter construct and a β -galactosidase plasmid. Basal NF- κ B transcriptional activity in 5-month MC were arbitrarily defined as 1. Data shown are from six measurements of each of four independent experiments. **A:** Basal NF- κ B transcriptional activity (base) was 2.6-fold higher in 28-month MC than in 5-month MC. The introduction of an exogenous IKK expression vector to 5-month MC or 28-month MC increased cell NF- κ B transcriptional activity. In contrast, transfection of 5-month or 28-month MC with an I κ B- α or dominant-negative IKK (dn) expression vector decreased cell NF- κ B transcriptional activity. **B:** Increased sensitivity to TNF- α induced NF- κ B activation in 28-month MC. Five ng/ml of TNF- α increased NF- κ B transcriptional activity in 28-month MC but not in 5-month MC. An increase in TNF- α levels to 10 ng/ml was required to stimulate NF- κ B activity in 5-month MC.

Figure 6. Inhibition of NF- κ B decreased RANTES and VCAM-1 expression in mesangial cells isolated from 28-month-old mice. Two mesangial cell lines isolated from 28-month-old B6 mice (28-month MC) were treated with a NF- κ B inhibitor, PDTC (0.075 μ mol/L), or transfected with an IKK dominant-negative expression vector (IKKDN) to suppress NF- κ B activity in cells. Baseline and TNF- α (5 ng/ml) induced RANTES and VCAM-1 expression in PDTC- or IKKDN-transfected 28-month MC were measured by competitive PCR. RANTES or VCAM-1 mRNA levels were normalized to GAPDH mRNA levels. The ratio of RANTES or VCAM-1 to GAPDH in mesangial cells isolated from 5-month-old mice (5-month MC) at baseline was arbitrarily defined as 100%. Data shown are from three independent experiments. **A:** PDTC treatment (0.075 μ mol/L for 8 hours) significantly reduced RANTES mRNA levels in 28-month MC (**, $P < 0.01$ versus 28-month MC at baseline). Treatment with vehicle (0.001% dimethyl sulfoxide) did not affect RANTES expression (data not shown). The induction of RANTES expression by TNF- α was completely inhibited by PDTC treatment (##, $P < 0.01$ versus TNF). The suppression of NF- κ B by transfecting 28-month MC with an IKKDN expression vector also significantly reduced RANTES mRNA levels in 28-month MC (*, $P < 0.05$ versus 28-month MC). Transfecting 28-month MC with an empty pCR3 vector did not affect RANTES expression. The introduction of IKKDN resulted in a decrease in TNF- α induced RANTES expression in 28-month MC (#, $P < 0.05$ versus TNF- α). **B:** PDTC treatment significantly reduced baseline and TNF- α -induced VCAM-1 expression in 28-month MC (**, $P < 0.01$ versus 28-month MC at baseline; ##, $P < 0.01$ versus TNF- α). Transfecting 28-month MC with an IKKDN expression vector, but not the pCR3 empty vector, decreased baseline and TNF- α -induced VCAM-1 expression in 28-month MC (*, $P < 0.05$ versus 28-month MC at baseline; #, $P < 0.05$ versus TNF- α).

cells from 5-month-old mice and completely blocked the effect of TNF- α (Figure 6). Transfection of mesangial cells from 28-month-old mice with IKKDN also significantly reduced RANTES and VCAM-1 expression. The effect of TNF- α on RANTES and VCAM-1 expression was decreased in mesangial cells transfected with IKKDN.

Increased Serum TNF- α Levels in 22- to 28-Month-Old Mice

Serum TNF- α levels were higher in 22- to 28-month-old (72.9 $\pm$ 15.9 pg/ml) compared to 3- to 9-month-old female B6 mice (59.33 $\pm$ 9.2 pg/ml, $P < 0.05$).

Discussion

The incidence of chronic renal failure increases in women after menopause.³ Because aging female B6 mice develop progressive glomerulosclerosis in the absence of recognizable associated diseases, in common with aging women in the United States, we selected this model to study the molecular mechanisms of glomerulosclerosis in aging.^{3,9,27,28} The glomerular gene expression profile in late postmenopausal B6 mice (28 months old) revealed increased expression of inflammation-related genes. Among 14 of these, 7 are expressed by monocytes/macrophages, consistent with the finding that the glomeruli contained many macrophages.⁹

The mechanism underlying the recruitment of macrophages to the glomeruli could be related to stable phe-

notypic changes, which we found in many progressive glomerular diseases, including aging.^{2,9,29–33} Characteristic changes included alterations of extracellular matrix turnover,^{9,30–32} an increase in mesangial cell proliferation,²⁹ and an increase in cell size.³⁰ Phenotypic changes identified in cultured mesangial cells were often present in intact glomeruli.^{34,35} The glomeruli of 28-month-old B6 mice exhibited sclerosis and inflammatory lesions and their mesangial cells had a sclerotic phenotype. Herein, we examined whether cells from these aged mice also expressed an inflammatory phenotype. Intact glomeruli of 28-month-old mice exhibited increased RANTES and VCAM-1 expression, their mesangial cells had elevated levels of RANTES and VCAM-1 mRNA. Thus, the increased RANTES and VCAM-1 expression observed in intact glomeruli of 28-month-old mice might partly originate from mesangial cells. Mesangial cells have been suggested to play an important role in glomerular leukocyte infiltration.^{16–18} RANTES is a chemokine that attracts T cells, monocytes, and other leukocytes.³⁶ CC chemokine receptor 5 (CCR5) is the receptor for RANTES. In this study we found that RANTES, but not CCR5 expression was increased in the glomeruli of 28-month-old mice. Increased RANTES expression has been found in glomerulonephritis with prominent monocyte/macrophage infiltration.^{37,38} Thus, the increased RANTES expression found in 28-month-old B6 mice mesangial cells could contribute to glomerular monocyte/macrophage infiltration.

VCAM-1 is a member of the immunoglobulin supergene family of receptors. The interaction of VCAM-1 with its ligand, very late antigen-4, results in strong cell-cell

adhesion, an essential step for leukocyte infiltration.³⁹ The presence of an increase in both VCAM-1 and RANTES in 28-month-old mice mesangial cells may be either additive or their dual presence could amplify glomerular monocyte/macrophage infiltration.

The cause of increased glomerular inflammatory reaction in 28-month-old (postmenopausal) mice is unknown. One possibility is that it is induced by inflammatory cytokines. In fact, the levels of circulating TNF- α or TNF- α expression by peripheral mononuclear cells have been reported to be increased in postmenopausal women and in ovariectomized premenopausal women.⁴⁰ In the current study we found that the serum TNF- α levels in 22- to 28-month-old postmenopausal B6 mice were increased. Because TNF- α has been shown to increase RANTES and ICAM-1 expression in premenopausal mesangial cells, the inflammatory phenotype present in mesangial cells of 28-month-old mice could have been induced by the elevated levels of TNF- α .¹⁶ In the present study we found that mesangial cells isolated from 28-month-old mice were more sensitive to the proinflammatory effects of TNF- α than were cells isolated from 5-month-old mice. Although 5 ng/ml of TNF- α induced up-regulation of RANTES and VCAM-1 expression in mesangial cells from 28-month-old mice, a twofold higher dose (10 ng/ml TNF- α) was required to induce a similar response in mesangial cells isolated from 5-month-old mice. Thus, both increased circulating TNF- α levels and a twofold increase in mesangial cell sensitivity to TNF- α may contribute to the inflammatory glomerular lesions in aging. Interestingly, we found that mesangial cells isolated from 18- to 22-month-old postmenopausal B6 mice also exhibited an increased response to TNF- α (data not shown), at a time that there were no inflammatory glomerular lesions. Thus, phenotypic changes in mesangial cells could be one of the initiators of glomerular macrophage infiltration.

TNF- α activity is mediated by two distinct cell surface receptors, TNFR1 and TNFR2, which are co-expressed on mesangial and most other cell types. Because there was no difference in TNFR1 or TNFR2 mRNA expression between mesangial cells from 5- and 28-month-old mice, it is unlikely that the increased TNF- α response in mesangial cells isolated from 28-month-old mice was because of changed levels of TNF- α receptor expression. TNFR1 mediates many proinflammatory effects of TNF- α .⁴¹ We found that baseline levels of RANTES and VCAM-1 expression were lower in mesangial cells lacking TNFR1, than in cells lacking TNFR2. TNF- α did not induce RANTES expression in mesangial cells lacking TNFR1, showing that this receptor mediates TNF- α signaling. However, VCAM-1 expression remained responsive to TNF- α in TNFR1-deficient cells. Because TNFR2-deficient mesangial cells had increased RANTES expression after TNF- α induction, the induction of RANTES expression by TNF- α appeared to be mediated by TNFR1, not by TNFR2. The up-regulation of VCAM-1 expression by TNF- α in TNFR1 or TNFR2-deficient mesangial cells indicated that signaling pathways other than these two receptors might be involved in TNF- α -induced VCAM-1 expression. Thus, the signaling pathways for the regulation of RANTES and VCAM-1 expression by TNF- α

in mesangial cells appeared to be distinct. However, it is not known if there is a cause-effect relationship between TNFR1 and TNFR2 signaling and increased glomerular inflammatory responses in aging.

Increased NF- κ B activity has been found in the kidney, liver, heart, and brain of aged animals.^{42–44} NF- κ B activation is responsible for the proinflammatory effects of TNF- α .^{41,45} We found that NF- κ B was functionally active and regulated by I κ B α and IKK in B6 mesangial cells. NF- κ B transcriptional activity was higher in mesangial cells isolated from 28-month-old mice than in those from 5-month-old mice. In addition, mesangial cells from 28-month-old mice were more sensitive to TNF- α -induced NF- κ B activation. Because the expression of RANTES and VCAM-1 in mesangial cells, and other cell types, is regulated by NF- κ B, we speculated that increased RANTES and VCAM-1 mRNA levels in mesangial cells from 28-month-old mice may be because of increased NF- κ B activity. The findings that the inhibition of NF- κ B activity by either PDTC or the overexpression of dominant-negative IKK in these cells decreased baseline and TNF- α -induced RANTES and VCAM-1 expression supported this possibility. The cause of increased NF- κ B transcriptional activity in mesangial cells from 28-month-old mice is not clear. However, an increase in circulating TNF- α levels, an increased sensitivity to TNF- α , and the presence of oxidative injury may contribute to the development and expression of this mesangial cell phenotype *in vivo* and *in vitro*.

Inflammation may be a common component of the aging process because a gene expression profile demonstrating increased inflammatory responses has been found in multiple organs such as the liver and brain of aged B6 mice.^{12–15} Thus, an increase in oxidative stress and in proinflammatory cytokine production, as well as the loss of sex hormones, may contribute to inflammatory lesions in aging.^{7,40,46}

In summary, we found that mesangial cells isolated from B6 mice in the late postmenopausal period exhibited complex phenotypic changes that were both pro-sclerotic and proinflammatory. They consisted of increased expression of type I and type IV collagen and RANTES and VCAM-1, elevated NF- κ B activity, and increased sensitivity to TNF- α . These changes may contribute to the glomerular extracellular matrix expansion and inflammatory lesions in aging.

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