Complement Activation in Angiotensin II–Induced Organ Damage


Abstract—We tested whether or not complement activation participates in angiotensin (Ang) II–induced vasculopathy. We used double transgenic rats harboring human renin and angiotensinogen genes (dTGR) with or without losartan or the human renin inhibitor aliskiren. Sprague–Dawley (SD) rats were controls. DTGR had increased blood pressure at week 5 that increased further by week 7. Albuminuria was absent at week 5 but increased markedly in weeks 6 and 7. C-reactive protein (CRP) elevation, macrophages, T cells, tumor necrosis factor (TNF)-α, C1q, C3, C3c, and C5b-9 expression preceded albuminuria. C1q, C3, C3c, and C5b-9 were observed in the dTGR vessel media. C5b-9 localized with interleukin (IL)-6. Losartan and aliskiren reduced albuminuria and complement expression. We also studied vascular smooth muscle cells (VSMC) from dTGR compared VSMC from SD. C3 and IL-6 mRNA were analyzed after Ang II, TNF-α, and CRP stimulation. VSMC from dTGR showed increased proliferation and C3 expression compared with SD. Ang II did not induce C3 mRNA in either VSMC type. However, TNF-α and CRP induced C3 mRNA slightly in SD VSMC but markedly in dTGR VSMC, whereas IL-6 induction was similar in both. Thus, complement activation and cell infiltration occurred before the onset of albuminuria in Ang II–mediated renal damage. TNF-α and CRP played a major role in C3 activation. VSMC from dTGR are more sensitive for C3 activation. Our data show that, in this Ang II–induced model, complement activation is a major participant and suggest that TNF-α and CRP may play a role in its induction. (Circ Res. 2005;97:716-724.)

Key Words: angiotensin II • complement • immune system • albuminuria and renal damage

The innate complement system eliminates invading pathogens, stimulates opsonization, enhances phagocytosis, cytolysis, chemotaxis, and solubilizes immune complexes. Complement forms a bridge between innate and acquired immunity.1,2 On excessive activation or inappropriate deposition, complement can cause disease.3 The classical alternative and lectin complement pathway merge at the level of C3, resulting in the generation of C5b-9, the membrane attack complex. Complement activation has been implicated in the pathogenesis of numerous proteinuric renal diseases including glomerulonephritis, transplant rejection, and ischemia-reperfusion injury.1–3 Pratt et al demonstrated that the absence of local C3 production modulates renal graft survival and regulates T-cell priming of donor antigens.4 Very recently, Lin et al reported that young spontaneous hypertensive rats (SHR) that have not yet developed hypertension showed increased C3 expression and increased vascular smooth muscle cell (VSMC) proliferation. Both were blocked by C3 downregulation.8 Several studies showed that angiotensin (Ang) II not only is a vasoconstrictor peptide but also promotes inflammation and renal damage. We showed recently that immunosuppression improved nonimmune Ang II–mediated renal damage.9 The evidence that Ang II affects the complement system is indirect. Abbate et al demonstrated that angiotensin-converting enzyme (ACE) inhibition reduced cell infiltration and C3 immunoreactivity in rats with 5 of 6 nephrectomy.10 To investigate complement in an Ang II–induced model, we first tested whether or not complement is activated. Second, we investigated whether or not inhibition of the renin–angiotensin system by 2 independent mechanisms would affect complement activation. Third, we asked whether or not complement activation occurs before or subsequent to renal damage. Fourth, we conducted in vitro signal-transduction experiments to elucidate participants in complement activation.

Materials and Methods

Experimental Design

We studied male transgenic rats harboring human renin and angiotensinogen genes (dTGR) (RCC Ltd; Basel, Switzerland) and age-
matched nontransgenic SD rats (Tierzucht Schönwalde, Germany). American Physiological Society guidelines for animal care were followed (permit no. G 237/03). Untreated dTGR (n=10 week 5 and 6; n=18 week 7) and SD (n=8 each group) rats were killed at age 5, 6, and 7 weeks. Losartan (LOS)-treated and the human renin inhibitor aliskiren (ALISK)-treated dTGR were killed at week 7. Four-week-old dTGR were treated with LOS (10 mg/kg in the diet, n=15) or ALISK (3 mg/kg per day SC by minipump infusion, n=11) for 3 weeks. Systolic blood pressure was measured weekly by tail cuff. Twenty-four-hour urine samples were collected in metabolic cages from weeks 5 to 7. Serum and plasma were collected at week 7. Urinary rat albumin and serum rat C-reactive protein (CRP) were determined by ELISA (CellTrend, Germany, and BD Pharmingen, Germany, respectively). Blood urea nitrogen and creatinine were determined by an automated clinical method.

**Immunohistochemistry, Confocal Microscopy, and Antibodies**

Ice-cold acetone-fixed cryosections (6 μm) were stained by immunofluorescence or alkaline phosphatase–anti-alkaline phosphatase techniques as described earlier.13 The sections were incubated with the following monoclonal antibodies: anti–ED-1 and anti–CD8 (Clone Ox-8) (both from Serotec, Düsseldorf, Germany); anti–CD4 (Clone Ox-38), anti–CD86, anti-Ox62, and anti–major histocompatibility complex (MHC) II (Clone Ox-6) (all BD Pharmingen); and anti–C5b-9 (2A1) (kindly provided by Dr W. Couser, University of Washington, Seattle). The following polyclonal antibodies were used: rabbit anti-rat IgG (DAKO, Hamburg, Germany); anti-fibronectin (Paesel, Frankfurt, Germany); anti–collagen IV (Southern Biotechnology, Birmingham, Ala); anti-C1q (A 0138; DAKO); fluorescein isothiocyanate–conjugated rabbit anti-C3 (ICN, Eschwege, Germany); fluorescein isothiocyanate–conjugated anti-rabbit anti-C5c complement (Roche Diagnostics, Mannheim, Germany); and goat anti-tumor necrosis factor (TNF)–α (Santa Cruz Biotechnologies, Heidelberg, Germany). Confocal microscopy was performed as described earlier.13 VSMC were stained with anti–smooth muscle actin (SM) α-actin (DAKO) and anti-vinculin (BioTrend, Germany) and analyzed by 2 different investigators (E.S. and J.-K.P.) without knowledge of the origin of the specimens.

**Quantification of Infiltrated Cells and Complement Expression**

Sections were analyzed with a Zeiss Axiosplan-2 microscope (Carl Zeiss) and AxioVision 2 multichannel image processing system (Carl Zeiss). Semiquantitative scoring of infiltrated (ED-1, CD4, CD8, Ox62, Ox6, CD86) cells, in 15 different cortical kidney areas (n=6 per group), was performed using computerized cell count program (KS 300 3.0; Carl Zeiss) with samples examined blind. For quantification of perivascular macrophage infiltration, all selected view fields included a small vessel in their analysis. Quantification of CD4+ T cells, MHC II+, and CD86+ cells was performed periglomerular, whereas CD8+ cells were quantified interstitially. The number of IgG-positive glomeruli was counted in 50 randomly selected glomeruli in different cortical areas. For quantitative analysis of complement components C1q, C3, C5c, and C5b-9, we counted the total number of positive-stained vessels and glomeruli and positive-stained group of tubules per whole-kidney cross-section based on the staining intensity.

**Cell-Culture Experiments**

Aortic VSMC were isolated from dTGR and SD rats as described previously.13 VSMC from passages 4 and 8 were treated with Ang II (10 to 7 mol/L; Sigma), TNF-α (10 ng/mL; Calbiochem), or human CRP (50 μg/mL; Calbiochem) for 24 hours. Rat tubular epithelial NRK-52E cells (NRK) were treated with Ang II and TNF-α. To analyze whether C3 was regulated in a nuclear factor κB (NF-κB)–dependent manner, we treated dTGR-VSMC in the absence and presence of the IκB phosphorylation inhibitor BAY 11 to 7085 (20 μmol/L; Calbiochem) and IκB kinase (IKK)-2 inhibitor se 514 (100 μmol/L; Calbiochem) with TNF-α for 3 hours. Cell-proliferation experiments were performed after a method described by Crouch et al.14 The proliferation was determined after 3 days and calculated as fold-induction per baseline.

**Quantitative TaqMan RT-PCR**

RNA isolation and TaqMan RT-PCR were performed as described previously.13 Each sample was in triplicate. For quantification, the target sequences were normalized in relation to the 18S product. Biotec (Berlin, Germany) synthesized the primers. The sequences are available on request.

**Electrophoretic Mobility Shift Assay**

VSMC from dTGR and SD were stimulated with TNF-α (10 ng/mL) for 15 minutes. Electrophoretic mobility shift assay was performed as described previously.14 Total cell extracts (5 μg) were incubated with oligonucleotides, containing the NF-κB–binding site from the MHC enhancer (H2K, 5′-gatCACGGGCTGGGAGTTCCCACTCTCCACAGG). In competition assays, 50 ng of unlabeled H2K oligonucleotides were used. For supershift assay, 1 μg of anti-p50 or anti-p65 (both from Santa Cruz Biotechnologies) were added for 20 minutes to the homogenates before addition of the labeled probe. The shifts (n=4) were quantitated with the NIH image program.

**Ancillary Experiments**

To support our observations, we conducted ancillary experiments with the methodology described above or reanalyzed specimens from earlier studies. These protocols and results are given in the online data supplement available at http://circres.ahajournals.org.

**Statistics**

Data are presented as means±SEM. Statistically significant differences in mean values were tested by ANOVA and blood pressure and albuminuria by repeated-measures ANOVA and the Scheffé test. A value of P<0.05 was considered statistically significant. The data were analyzed using Statview statistical software.

**Results**

**Blood Pressure and Renal Damage**

Untreated 5-week-old dTGR showed moderately elevated systolic blood pressure compared with age-matched SD rats (138±4 versus 91±5 mm Hg), which progressively increased over time to week 7 (204±5 mm Hg). LOS treatment reduced blood pressure (141±6 mm Hg) partially, whereas ALISK normalized blood pressure (111±2 mm Hg) to SD values (119±6; Figure 1A). In contrast, albuminuria, serum creatinine, and blood urea nitrogen were not different at week 5 between dTGR and SD. Increased albuminuria was first observed at week 6 and reached a 150-fold increase at week 9 (Figure 1B). LOS and ALISK treatment reduced albuminuria by 85% and 99%, respectively. SD rats showed no albuminuria. Blood urea nitrogen levels were 13±0.5 versus 12±0.4 at week 5 and 17±1 versus 13±1 mg/dL at week 6 but significantly elevated at week 7 (65±15 versus 13±0.4 mg/dL dTGR versus SD, respectively). LOS- and ALISK-treated dTGR showed normal levels at week 7 (16±0.4 and 16±0.7 mg/dL, respectively). Serum creatinine was not changed at weeks 5 and 6 but significantly increased at week 7 (Figure 1C). Both LOS and ALISK reduced serum creatinine significantly (Figure 1C). Renal fibrosis (fibronecin and collagen IV) was first detected at week 6 and markedly increased at week 7 in untreated dTGR and was abolished by LOS and ALISK treatment (data not shown).
DTGR Show Increased Serum C-Reactive Protein, Renal TNF-α, and Cell Infiltration

Serum CRP in dTGR was increased 10-fold at week 5 compared with SD (51±5 versus 4±1 mg/mL). CRP reached 66.0±5.7 mg/mL at week 7. LOS and ALISK treatment reduced CRP by 90% and 88% to SD levels (Figure 1D). We next analyzed the proinflammatory cytokine TNF-α in the kidney. We detected elevated immunoreactivity of TNF-α in untreated dTGR at week 5 that progressively increased over time. TNF-α was detected in glomeruli, in the media of vessel walls, and in tubular epithelial cells (brush borders). Age-matched SD showed no staining. TNF-α was partially reduced in LOS-treated dTGR and completely reduced in ALISK-treated dTGR (Figure 2). Because TNF-α promotes inflammation, we next focused on renal-cell infiltration. Monocytes/macrophages (ED-1+ cells) infiltrated predominantly around the damaged vessels; CD4+ T-helper, CD86+, and MHC II+ (OX-62+) cells showed a perivascular and interstitial location; cytotoxic CD8 T cells and dendritic cells were at interstitial, periglomerular, and glomerular locations. Semiquantification revealed that all cell types, except dendritic cells, were increased in untreated dTGR already at week 5, before the onset of albuminuria. LOS and ALISK treatment prevented the infiltration of all cell types (Figure 3), with 1 exception. CD4+ T cells were only partially reduced by LOS.

Renal Expression of Complement Components Precedes the Onset of Albuminuria

In contrast to cell infiltration, the role of Ang II on complement components C1q, C3, C3c, and the membrane attack complex C5b-9 is not well analyzed. Figure 4A shows increased C1q immunostaining in the media of 5-week-old dTGR compared with age-matched SD. There were only a few C1q-positive glomeruli at week 7 by dTGR (data not shown). Age-matched SD control and LOS- and ALISK-
Renal TNF-α Expression

![Renal TNF-α Expression](image)

**Figure 2.** Renal TNF-α expression. DTGR kidneys show increased TNF-α immunoreactivity (red) in glomeruli, in the media of vessel wall, and in tubular epithelial cells dominantly on tubular brush borders. TNF-α immunostaining was detected already at week 5 in DTGR and increased over time. SD showed no TNF-α staining. LOS and ALISK treatment reduced it. The autofluorescence (green) was used to demonstrate the kidney structure.

treated rats showed no staining for C1q. Semiquantification of C1q demonstrated that the number of C1q-positive vessels in DTGR increased over time and was higher compared with SD at all time points. Already at week 5, DTGR showed C3 immunoreactivity in tubular epithelial cells, in the media of small vessels, and granular staining in glomeruli (Figure 4B). The intensity and the accumulation of C3 staining increased over time. We also analyzed the active cleavage product C3c (Figure 4C). Renal C3c expression was observed in the media of small vessels in 5-week-old DTGR, whereas C3c expression on the brush borders of tubular epithelial cells was first detected at week 6. Semiquantification confirmed a higher number of C3 and C3c staining in DTGR compared with other groups (Figure 4D). The immunostaining for the membrane attack complex C5b-9 resembled the pattern of C3. The number of C5b-9–positive vessels, tubules, and glomeruli in untreated DTGR was significantly increased compared with SD. LOS and ALISK both reduced renal C5b-9 immunoreactivity (Figure 5A and 5B). C5b-9 is able to induce interleukin (IL)-6. We, thus, performed costainings. The media of small untreated DTGR vessels showed expression of both C5b-9 and IL-6 (Figure 5C). However, IL-6 was also expressed in the neointima of damaged DTGR vessels.

**VSMC From DTGR Are More Sensitive for C3 Activation**

VSMC isolated from DTGR showed increased cell proliferation, decreased SM α-actin and vinculin immunostaining, and increased C3 mRNA expression compared with SD VSMC, indicating a switch from the contractile to a synthetic phenotype (Figure 6A). Ang II treatment did not induce C3 mRNA in either SD or DTGR VSMC (Figure 6B). TNF-α (Figure 6B) and CRP (Figure 6C) induced C3 mRNA slightly above baseline in the SD VSMC but massively in the DTGR VSMC. In contrast, IL-6 was induced to a similar extent in both VSMC types (Figure 6B and 6C). We also investigated whether inhibition of the IKK–NF-κB pathway would prevent TNF-α–induced C3 activation. The IkBa phosphorylation inhibitor BAY 11-8702 and the IKK-2 inhibitor sc 514 inhibited TNF-α–mediated C3 induction in DTGR VSMC (Figure 6D). Finally, we found that TNF-α induced NF-κB DNA-binding activity to a greater extent in VSMC from DTGR compared with SD (Figure 6E).

We next analyzed the role of Ang II and TNF-α on C3 in renal tubular epithelial cells (NRK). We found only C3 and IL-6 induction after TNF-α (1144±215 and 3.1±0.4 arbitrary units for C3 and IL-6, respectively), but not after Ang II (7±1 and 2.2±0.2 arbitrary units for C3 and IL-6, respectively), compared with unstimulated cells (10±2 and 1.7±0.3 arbitrary units for C3 and IL-6, respectively).

**Discussion**

We provide the first evidence that complement components are activated in a model of Ang II–induced renal damage. There were 4 major findings. First, complement C1q, C3, C3c, and C5b-9, as well as increased CRP, renal TNF-α, and cell infiltration, occurred before the onset of albuminuria and were reduced by Ang II type 1 receptor blockade or by the human renin inhibitor ALISK. Second, our in vitro experiments in VSMC and in renal tubular epithelial cells showed that Ang II did not directly induce C3. Third, CRP and TNF-α both induced a more pronounced C3 activation in VSMC from DTGR compared with SD VSMC. NF-κB DNA-binding activity in DTGR VSMC was higher compared with SD VSMC after TNF-α induction. Finally, TNF-α–induced C3 stimulation was blocked by IKK–NF-κB inhibition. Taken together, CRP and TNF-α play an important role in C3 activation in vitro and in vivo.

The complement activation in immune-mediated renal diseases has been appreciated for decades. Nonetheless, not much information is known about the role of Ang II in complement activation. Abbate et al provided indirect evidence that Ang II leads to complement activation.10 They showed that chronic ACE inhibitor treatment reduced complement C3 deposition in the remnant kidney model. From their study, whether or not the reduction of C3 was attribut-
able to decreased Ang II levels or to the improved renal function resulting from the ACE inhibitor treatment is not clear. We, therefore, addressed the question of whether complement activation precedes or is the consequence of albuminuria in Ang II–induced renal damage. Our data provide clear evidence that the complement components C1q, C3, C3c, and C5b-9 are activated and that infiltration of macrophages and T cells occurs before the albuminuria commences. Both inflammation and complement activation probably contribute to the renal disease progression. We observed complement C3 and C3c immunostaining in the vessel wall and tubules. Complement activation is mainly initiated by cleavage of C3 to C3b. C3b then binds to cell membranes, leading to the generation of C3 and C5 convertase, unless C3b is cleaved to inactive C3bi. Thereafter, C3bi is cleaved by factor I to C3c and C3d. Numerous findings indicate that glomerular C3c deposition reflects very recent complement activation. In IgA nephropathy, glomerular C3c deposition disappeared to normal values in parallel with the return of elevated urinary C5b-9 excretion. We investigated whether complement activation was a specific finding in our dTGR model or whether complement activation is a general feature of Ang II–induced renal damage. We infused SD rats with Ang II. Furthermore, we analyzed a completely different transgenic model, namely rats overexpressing the mouse renin-2 gene. Both models showed elevated blood pressure, albuminuria, CRP, C1q, C3, C3c, and C5b-9, indicating that our findings were not limited to the dTGR model (online data supplement).

To differentiate whether C3 activation is directly mediated by Ang II or is the consequence of hypertension and/or renal damage, we analyzed sections from earlier studies on dTGR. Dexamethasone-treated dTGR were severely hypertensive with blood pressure levels >200 mm Hg; however, dexamethasone treatment reduced mortality to 0 and markedly lowered albuminuria. The newly stained histological sec-
tions from the dexamethasone-treated dTGR showed no complement. None of the complement components were identified (online data supplement). We also had available specimens from an earlier study in which we treated dTGR with a triple therapy of non–renin–angiotensin system inhibitors (reserpine, hydralazine, and hydrochlorothiazide). The rats from that study developed albuminuria and renal complement component expression in a similar fashion as untreated dTGR, despite normal blood pressure values (online data supplement).

We also performed cell-culture experiments in the current study. We found no evidence of direct Ang II–mediated C3 induction in the cell types at the time points (3, 6, 24, 48, and 72 hours) tested. One limitation of our in vitro experiments was the fact that the VSMC were isolated from aorta and not from small renal vessels. However, the results were nonethe-

less striking. Ang II exposure did little to C3 mRNA but induced IL-6 in SD VSMC. Because in dTGR, the VSMC are chronically exposed to Ang II, we suspected that in vivo Ang II induces local mediators like TNF-α. In contrast to Ang II, CRP and TNF-α activated C3, both in VSMC and tubular epithelial cells. Our in vitro findings and data from Lin et al, who demonstrated C3 activation in VSMC from prehypertensive SHR rats, suggest that high blood pressure is not the main reason for complement activation. Lin et al termed VSMC from control rats as contractile and VSMC from SHR as synthetic based on differences in growth rates and gene expression. We observed that dTGR VSMC showed typical features of the synthetic phenotype with increased cell proliferation, decreased SM α-actin and vinculin expression, and a 4-fold C3 mRNA expression. Lin et al also demonstrated that exogenous C3 leads to the synthetic VSMC phenotype.
In our study, the 4-fold higher C3 levels might have contributed to the VSMC phenotype switch. The dTGR VSMC were far-more sensitive to CRP and TNF-α compared with SD VSMC, which agrees with the findings of Lin et al. These investigators also provided an interesting new C3 function by demonstrating that C3 inhibition resulted in decreased cell proliferation. Our VSMC data are the first indicating that IKK–NF-κB inhibition prevents C3 activation. In addition, we found that the synthetic phenotype is more susceptible to TNF-α-induced NF-κB DNA-binding activity induction. Nevertheless, CRP and TNF-α stimulation induced IL-6 to a similar extent in the synthetic and contractile VSMC, which speaks for the involvement of alternative regulatory pathways for C3 and IL-6.

In vitro, TNF-α appeared to play a major role in C3 activation. We reported recently that TNF-α blockade ameliorates renal damage in our transgenic model. To clarify whether or not in vivo TNF-α blockade affects renal C3 expression, we reexamined the histological sections from that study. We found that TNF-α blockade markedly reduces complement C1q and C3 activation (online data supplement). These results underscore the importance of TNF-α in the innate immunity that occurs in this model.

The C5b-9 membrane attack complex forms pores in cells, resulting in cell activation or, at higher concentration, leading to cell death by lysis. Experiments in complement-deficient mice provided evidence that C5b-9 is involved in deoxycorticosterone acetate salt–induced hypertension-mediated renal damage, nonimmune remnant kidney model, and renal ischemia reperfusion injury. In vitro experiments showed that C5b-9 induces IL-6 and TNF-α synthesis in tubular epithelial cells. Viedt et al showed that C5b-9 induced IL-6...
in VSMC through the activation of NF-κB and activator protein-1. We found that C5b-9 and IL-6 colocalized in damaged small dTGR vessels in VSMC. Interestingly, we observed only IL-6 immunoreactivity, but no C5b-9, in the neointima of these vessels.

Whether CRP is only a biomarker for inflammation or participates adversely in the pathogenesis of cardiovascular disease is a matter of debate. CRP is a predictor for myocardial infarction, stroke, metabolic syndrome, peripheral vascular disease, and vascular mortality among individuals with no known cardiovascular disease. In rodent models, CRP is involved in the pathogenesis of myocardial infarction, causes a higher rate of thrombotic occlusions, and mediates atherosclerosis in apolipoprotein E gene deficient mice. In isolated VSMC, CRP is able to activate NF-κB and activator protein-1 and increase Ang II type 1–receptor expression and binding-site, migration and proliferation. We focused on CRP-mediated C3 and IL-6 activation in the synthetic and contractile VSMC phenotypes. CRP activated IL-6 in both phenotypes to a similar extent, whereas CRP markedly induced C3 in the synthetic VSMC phenotype. In human coronary endothelial cells, CRP activated membrane cofactor protein-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1. In our model, circulating CRP was elevated long before any evidence for vascular injury. Possibly, Ang II sensitized the vasculature to effects of CRP, TNF-α, and other mediators. We believe that the role of CRP and complement might have been underestimated in Ang II–related hypertension. We showed the utility of renin–angiotensin system blockade in these experiments. However, additional mechanistic experiments are necessary. Examples include use of complement component (C5A inhibitors are available) inhibitors or studies in gene-deficient mice. Nevertheless, our observations are novel and suggest additional avenues to ameliorate Ang II–induced target-organ damage.

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Figure 6. Synthetic VSMC phenotype is more susceptible for C3 induction. A, VSMC from dTGR showed a higher proliferation rate (left), decreased SM α-actin and vinculin expression (middle), and 4-fold increased C3 mRNA levels (right). B and C, TNF–α and CRP induced C3 mRNA slightly in the contractile VSMC phenotype but more pronounced in the synthetic VSMC phenotype. IL-6 was induced to a similar extent in both VSMC phenotypes. Ang II did not induce C3 mRNA in either phenotype but induced IL-6 in the contractile VSMC phenotype. D, tκB phosphorylation and IKK inhibitor prevented TNF–α–induced NF-κB DNA-binding activity. E, Electrophoretic mobility shift assay showed that TNF–α–induced NF-κB DNA-binding activity was significantly more increased in the synthetic compared with the contractile VSMC phenotype. Arrows indicate p50 and p65 supershift. Quantification is shown in F. Results are mean±SEM. *P<0.05 vs unstimulated control, #P<0.05 vs unstimulated SD VSMC, ‡P<0.05 vs TNF–α or CRP–stimulated SD VSMC.
References

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Results on-line supplement

We performed additional studies to separate Ang II-related effects from blood pressure-related actions in our model. We stained kidney sections from dTGR treated with triple therapy (hydralazine, hydrochlorothiazide and reserpine) or with dexamethasone (DEXA) from our earlier studies on dTGR. DEXA-treated dTGR were severely hypertensive (blood pressure levels >200 mm Hg). However, DEXA reduced mortality to zero in this model and markedly lowered albuminuria. The histological sections from the DEXA-treated dTGR showed no complement. None of the complement components were identified. We also had available specimens from an earlier study in which we treated dTGR with a “triple therapy” of non-renin-angiotensin system inhibitors. The rats from that study developed albuminuria and expressed renal complement components in a similar fashion as untreated dTGR, despite normal blood pressure values. Renal complement C3 expression is shown in figure 1 supplement. Therefore, we conclude that hypertension is not the major cause for complement activation in this model.

We suspected that TNF-α might have played an important role in complement activation. Therefore, we stained kidney sections from dTGR treated with the TNF-α blocker, eternacept, for C1q and C3. Both complement components were significantly reduced, compared to controls as shown in figure 2 supplement.

We also investigated whether complement activation was a specific finding in our dTGR model or whether complement activation is a general feature of Ang II-induced renal damage. We investigated SD rats infused with Ang II as well as transgenic rats over expressing the mouse renin-2 gene (TGR(mREN-2)27), a transgenic model that also depends on Ang II. Similar to our dTGR model, we found elevated blood pressure, albuminuria, renal cell infiltration, CRP release, and the activation of complement components, as shown in figure 3 supplement. Systolic blood pressure and albuminuria were similar in TGR(mREN)27 and dTGR, but slightly lower in Ang II-infused SD rats. In contrast, CRP levels were similar in all three models. All three models showed C1q, C3, and C5b-9 complement activation. Nevertheless, there were some minor differences between the models. Glomerular C5b-9 was more highly expressed in TGR(mREN)27 compared to dTGR and Ang II-infused SD. In contrast, TGR(mREN)27 and Ang II-infused SD rats showed fewer C1q and C3 positive vessels compared to dTGR. Tubular and glomerular C3 was similar in all three models.
Figure legends

**Fig. 1 supplement.** Untreated and triple therapy (TRIPLE)-treated dTGR showed renal complement C3 expression. In contrast, dexamethasone (DEXA)-treated dTGR were C3 negative.

**Fig. 2 supplement.** Semi-quantification of C1q and C3 in untreated and etanercept-treated dTGR is shown. Both complement components were significantly reduced by etanercept treatment.

**Fig. 3 supplement.** A. Systolic blood pressure, albuminuria and C-reactive protein (CRP) are elevated in TGR(mREN)27 and Ang II-infused SD rats compared to SD controls. B. Complement C1q, C3 and C5b-9 were significantly more expressed in TGR(mREN)27 and Ang II-infused SD rats compared to SD controls.

**Supplement References**


Fig. 1 supplement

Renal Complement C3 Expression

dTGR

dTGR+DEXA

dTGR+TRIPLE
Fig. 2 supplement

Number of C1q+ Vessels per Kidney Cross Section

- dTGR
- dTGR+ etanercept

Number of C3+ Vessels per Kidney Cross Section

- dTGR
- dTGR+ etanercept

Number of C3+ Glomeruli per Kidney Cross Section

- dTGR
- dTGR+ etanercept

Number of C3+ Tubules per Kidney Cross Section

- dTGR
- dTGR+ etanercept

* denotes a statistically significant difference.
Fig. 3 supplement

A

Systolic Blood Pressure (mm Hg)

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Albuminuria (mg/d)

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C-reactive Protein (mg/d)

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B

Complement C1q

- TGR(mREN-2)
- SD+Ang II
- SD

Complement C3

- TGR(mREN-2)
- SD+Ang II
- SD

Complement C5b-9

- TGR(mREN-2)
- SD+Ang II
- SD