Role of the TNF pathway in the progression of diabetic nephropathy in KK-A\(^{\nu}\) mice

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Am J Physiol Renal Physiol 306: F1335–F1347, 2014. First published March 19, 2014; doi:10.1152/ajprenal.00509.2013.—Chronic inflammation promotes the progression of diabetic nephropathy (DN). However, the role of TNF-\(\alpha\) remains unclear. The objectives of the present study were to examine whether TNF-\(\alpha\) inhibition with a soluble TNF receptor (TNFR2) fusion protein, i.e., etanercept (ETN), improves the early stage of DN in the type 2 diabetic model of the KK-A\(^{\nu}\) mouse and to also investigate which TNF pathway, TNFR1 or TNFR2, is predominantly involved in the progression of this disease. ETN was injected intraperitoneally into mice for 8 wk. Renal damage was evaluated by immunohistochemistry, Western blot analysis, and/or real-time PCR. In vitro, mouse tubular proximal cells were stimulated by TNF-\(\alpha\) and/or high glucose (HG) and treated with ETN. ETN dramatically improved not only albuminuria but also glycemic control. Renal mRNA and/or protein levels of TNFR2, but not TNF-\(\alpha\) and TNFR1, in ETN-treated KK-A\(^{\nu}\) mice were significantly decreased compared with untreated KK-A\(^{\nu}\) mice. mRNA levels of ICAM-1, VCAM-1, and monocyte chemoattractant protein-1 and the number of F4/80-positive cells were all decreased after treatment. Numbers of cleaved caspase-3 and TUNEL-positive cells in untreated mice were very few and were not different from ETN-treated mice. In vitro, cleaved caspase-3- and TUNEL-positive cells in untreated mice were F4/80-positive cells were all decreased after treatment. Numbers of F4/80-positive cells were all decreased after treatment. Numbers of cleaved caspase-3 and TUNEL-positive cells in untreated mice were very few and were not different from ETN-treated mice. In vitro, cleaved caspase-3 and TUNEL-positive cells in untreated mice were very few and were not different from ETN-treated mice.

Tumor necrosis factor-\(\alpha\); tumor necrosis factor receptor; diabetic nephropathy; etanercept

DIABETIC NEPHROPATHY (DN) is a microvascular complication in type 2 diabetes (T2D). Recently, chronic subclinical inflammation has been thought to be involved in the pathogenesis of DN (4, 39). In particular, TNF-\(\alpha\) is cytotoxic to renal cells and able to induce direct renal injury (8). In fact, intrinsic renal cells, including mesangial, glomerular, endothelial, and renal tubular cells, are able to produce these cytokines (17, 28, 37, 46, 51). Experimental studies (8, 15, 37, 38, 46) have consistently reported that TNF-\(\alpha\) mRNA and protein levels are increased in glomerular and proximal tubular cells of diabetic rats. Other studies (15, 16, 38) have also demonstrated a significant role for TNF-\(\alpha\) in the development of renal hyperfiltration and hyperfiltration, two main alterations during the early stage of DN. Another relevant TNF-\(\alpha\) effect is the induction of apoptosis (9, 31). A recent experimental study (29) using isolated rat glomeruli demonstrated that this cytokine activates NADPH oxidase through PKC/phosphatidylinositol 3-kinase and MAPK pathways. Therefore, TNF-\(\alpha\), independent of hemodynamic factors, prompts the local generation of ROS, resulting in alterations in the barrier function of glomerular capillary walls and leading to enhanced albumin permeability (29). Strategies that inhibit TNF-\(\alpha\) have been successfully used in experimental diabetes. DiPetrillo et al. (15) reported that treatment of diabetic rats with an anti-TNF-\(\alpha\) agent, TNF receptor (TNFR)-Fc, reduced urinary TNF-\(\alpha\) excretion and prevented Na\(^{+}\) retention and renal hypertrophy. Similarly, TNF-\(\alpha\) inhibition with infliximab, a chimeric monoclonal antibody directed against TNF-\(\alpha\), significantly reduced both albuminuria and urinary TNF-\(\alpha\) excretion in streptozotocin-induced diabetic rats (36). However, the mechanisms by which anti-TNF-\(\alpha\) treatment decreases albuminuria is still unclear.

The KK-A\(^{\nu}\) mouse was established in 1969, and these mice are widely used as an experimental model for T2D (41). The KK-A\(^{\nu}\) mouse spontaneously exhibits T2D, associated with hyperglycemia, glucose intolerance, hyperinsulinemia, obesity, and microalbuminuria, although this mouse does not exhibit hypertension and renal dysfunction. Renal lesions in KK-A\(^{\nu}\) mice closely resemble those in human DN, with the glomeruli exhibiting diffuse mesangial hyperplasia with mesangial cell proliferation and segmental sclerosis. We and others (12, 27) have reported that the KK-A\(^{\nu}\) mouse is a suitable model for the study of early stage of DN in humans.

The objectives of the present study were 1) to investigate the therapeutic benefits of continuous neutralization of endogenous TNF-\(\alpha\) using a TNF-\(\alpha\) inhibitor, etanercept (ETN), in a mouse model of T2D and 2) to also investigate which TNF pathway, TNFR1 or TNFR2, is more critical for the progression of the early stage of DN in KK-A\(^{\nu}\) mice.

MATERIALS AND METHODS

Experimental animals and protocols. Seven-week-old male diabetic KK-A\(^{\nu}\) mice were purchased from CLEA Japan (Tokyo, Japan). We also purchased male KK mice (CLEA Japan) of the same age as controls for KK-A\(^{\nu}\) mice. These mice were individually housed in plastic cages with free access to food (rodent pellet diet NMF, 348 kcal/100 g, containing 5.5% crude fat) and water throughout the experimental periods. After being acclimatized to the new surroundings for 1 wk, mice were divided into the following three groups: 1) KK-A\(^{\nu}\) mice injected with distilled water (DW); untreated mice, \(n = \ldots\)

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11), 2) ETN-treated KK-A^y mice (ETN-treated mice, n = 20), and 3) KK mice injected with DW (n = 6). ETN (Enbrel, Wyeth Hellas SA, Athens, Greece) was injected intraperitoneally 2 times/wk at a dose of 1.0 mg/kg -1 day -1 into ETN-treated mice for 8 wk. The same amount of DW was injected intraperitoneally 2 times/wk to KK and untreated KK-A^y mice for the same duration. The experimental protocol was terminated when mice reached 16 wk of age. The dose of ETN was considered to be effective for the inhibition of TNF-α based on previous studies in mice (23, 48). All mice were maintained in the same room under conventional conditions with a regular 12:12-h light-dark cycle and were temperature controlled at 24 ± 1°C. All experiments were performed according to guidelines of the Animal Care Committee of Juntendo University.

Biochemical parameters in mice at 8 and 16 wk of age

<table>
<thead>
<tr>
<th></th>
<th>Nondiabetic KK Mice</th>
<th>Untreated KK-A^y Mice</th>
<th>ETN-treated KK-A^y Mice</th>
<th>Number of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 wk of age</td>
<td></td>
<td></td>
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<tr>
<td>Body weight, g</td>
<td>32.3 ± 0.8</td>
<td>37.8 ± 0.5</td>
<td>36.6 ± 1.8</td>
<td>6</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>168 ± 18</td>
<td>324 ± 105</td>
<td>300 ± 116</td>
<td>11</td>
</tr>
<tr>
<td>Hemoglobin A1c, %</td>
<td>4.3 ± 0.1</td>
<td>4.6 ± 0.3</td>
<td>4.4 ± 0.1</td>
<td>20</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>100 ± 10</td>
<td>110 ± 7</td>
<td>112 ± 3</td>
<td></td>
</tr>
<tr>
<td>Albumin-to-Cr ratio, mg/g Cr</td>
<td>22 (19, 27)</td>
<td>200 (117, 296)</td>
<td>176 (92, 222)</td>
<td></td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase, U/mg Cr</td>
<td>2.5 (1.8, 4.2)</td>
<td>2.2 (1.6, 4.6)</td>
<td>2.2 (2.9, 4.2)</td>
<td></td>
</tr>
<tr>
<td>8-Hydroxy-2′-deoxyguanosine, ng/mg Cr</td>
<td>20 (7, 72)</td>
<td>49 (35, 66)</td>
<td>59 (54, 66)</td>
<td>20</td>
</tr>
<tr>
<td>16 wk of age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>35.5 ± 1.9</td>
<td>49.5 ± 1.5</td>
<td>48.3 ± 2.3</td>
<td>6</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>147 ± 18</td>
<td>500 ± 83</td>
<td>351 ± 117</td>
<td>11</td>
</tr>
<tr>
<td>Hemoglobin A1c, %</td>
<td>4.1 ± 0.2</td>
<td>8.9 ± 1.4</td>
<td>7.6 ± 0.6</td>
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</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>112 ± 7</td>
<td>123 ± 4</td>
<td>118 ± 7</td>
<td>6</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
<td>105 ± 32</td>
<td>134 ± 28</td>
<td>129 ± 28</td>
<td>11</td>
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<tr>
<td>Tryglyceride, mg/dl</td>
<td>244 ± 57</td>
<td>366 ± 101</td>
<td>368 ± 100</td>
<td>20</td>
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<tr>
<td>Adiponectin, µg/ml</td>
<td>13.9 (13.2, 16.1)</td>
<td>9.9 (8.7, 11.0)</td>
<td>9.9 (8.3, 11.9)</td>
<td>6</td>
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<tr>
<td>Albumin-to-Cr ratio, mg/g Cr</td>
<td>23 (18, 28)</td>
<td>1,086 (942, 1310)</td>
<td>521 (202, 767)</td>
<td>11</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase, U/mg Cr</td>
<td>3.7 (2.7, 4.6)</td>
<td>19.5 (11.7, 31.6)</td>
<td>9.8 (3.7, 19.6)</td>
<td>20</td>
</tr>
<tr>
<td>8-Hydroxy-2′-deoxyguanosine, ng/mg Cr</td>
<td>30 (10, 49)</td>
<td>68 (52, 76)</td>
<td>54 (38, 63)</td>
<td>20</td>
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Data are expressed as means ± SD or as medians (25th percentile, 75th percentile). ETN, etanercept; Cr, creatinine.
Fig. 1. Immunohistochemical staining of TNF-α, TNF receptor (TNFR)1, and TNFR2 in mouse renal sections. Immunohistochemical staining of TNF-α (A–F), TNFR1 (H–M), and TNFR2 (O–T) in mice at 16 wk of age. Nondiabetic KK mice (A, D, H, K, O, and R), untreated (no-treated) KK-Ay mice (B, E, I, L, P, and S), etanercept (ETN)-treated KK-Ay mice (C, F, J, M, Q, and T). Images were taken at 40-fold (A–C, H–J, and O–Q) and 400-fold (D–F, K–M, and R–T) magnification. WIA indicates the whole interstitial area. TNF-α-stained areas in the renal tubulointerstitium in untreated KK-Ay mice were significantly larger than those in KK mice, but those levels did not differ between ETN-treated and untreated KK-Ay mice (G). TNFR1-positive cells were mainly localized in the proximal tubules, and those levels did not differ among three groups (N). TNFR2-stained areas in untreated KK-Ay mice were significantly larger than those in KK mice. Levels in ETN-treated KK-Ay mice were significantly smaller than those in untreated KK-Ay mice (U).
Apoptotic cells were quantified by counting the number of TUNEL-positive cells against whole kidney area, and the average number per whole kidney was determined.

Quantitative analysis of TNF-α/H9251, TNFR1, TNFR2, ICAM-1, VCAM-1, monocyte chemoattractant protein-1, RIPK1, RIPK3, and mixed lineage kinase domain-like mRNA levels by real-time PCR. Total RNA was extracted from whole kidneys of mice at 16 wk of age using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). RNA was reverse transcribed using random decamer primers (Ambion, Austin, TX) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Carlsbad, CA). TaqMan real-time PCR was performed, and results were analyzed according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). The primers used were as follows: TNF-α, Mm-00443258-m1; TNFR1, Mm-01182929-m1; TNFR2, Mm-00441889-m1; ICAM-1, Mm-00516023-m1; VCAM-1, Mm-00441242-m1; monocyte chemoattractant protein (MCP)-1, Mm-00441242-m1; RIPK1, Mm-00436354-m1; RIPK3, Mm-01319233-g1; mixed lineage kinase domain-like (MLKL), Mm-01244219-m1; and GAPDH, Mm-99999915-g1. The relative mRNA level in the sample was normalized for GAPDH content.

Western blot analysis of RIPK3 and MLKL expression. Portions of renal cortex samples were homogenized in lysis buffer containing a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Blots were probed with antibodies against RIPK3, MLKL, and GAPDH. Densitometric analysis was performed using ImageJ software.

Fig. 2. Renal mRNA levels of TNF-α, TNFR2, ICAM-1, VCAM-1, and monocyte chemoattractant protein (MCP)-1 in untreated KK mice, untreated KK-Ay mice, and ETN-treated KK-Ay mice at 16 wk of age. Renal mRNA levels of TNF-α, TNFR2, ICAM-1, and VCAM-1 in untreated KK-Ay mice were significantly higher than those in KK mice. mRNA levels of TNFR2 in ETN-treated KK-Ay mice were significantly decreased compared with those in untreated KK-Ay mice. mRNA levels of TNF-α and TNFR1 did not differ between ETN-treated and untreated KK-Ay mice. mRNA levels of ICAM-1, VCAM-1, and MCP-1 were also significantly decreased after treatment with ETN. *P < 0.05 vs. KK mice; †P < 0.05 vs. untreated KK-Ay mice.

Fig. 3. Immunohistochemical staining of F4/80 in mouse renal sections at 16 wk of age. A and D: nondiabetic KK mice; B and E: untreated KK-Ay mice; C and F: ETN-treated KK-Ay mice. The number of F4/80-positive cells was counted to determine macrophage infiltration in each group. The number of F4/80-positive cells in the tubulointerstitium of untreated KK-Ay mice was significantly increased compared with that in KK mice. The number of F4/80-positive cells was also significantly decreased after treatment with ETN (G). Images were taken at 40-fold (A–C) and 200-fold (D–F) magnification. *P < 0.05 vs. KK mice; †P < 0.05 vs. untreated KK-Ay mice.
used for the assay. The reaction was started by the addition of 40 μM of the fluorophoric QXL520/FAM FRET substrate. Fluorescence of the cleavage product was measured in a fluorescence microplate reader (FLx800, BIO-TEK Instruments, Winooski, VT) at an excitation of 490 nm and emission of 520 nm.

**Cell culture and reagents.** Cultured mouse proximal tubular (mProx) cells were kindly provided by Dr. Sugaya (CIMIC, Tokyo, Japan). Cells were passaged once every other day in medium [DMEM with high glucose (HG; 25 mmol/l); HG-DMEM] with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator containing 5% CO2 at 37°C (41). Cells were seeded in 10-cm culture dishes (1 × 10^6 cells/dish) and cultured overnight in growth medium, which was then replaced with low-glucose (LG; 5.5 mmol/l) medium (LG-DMEM), an osmotic control medium [LG-DMEM (5.5 mmol/l) + mannitol (25 mmol/l)], or HG-DMEM with 0.5% FCS at 12 h before the experiment. After 12 h, recombinant mouse TNF-α (R&D Systems, Minneapolis, MN) was added to the medium at a final concentration of 1 ng/ml with or without cotreatment with ETN (100 ng/ml). Cells in 10-cm culture dishes were harvested for extraction of mRNA after 48 h. Real-time PCR was performed to evaluate TNFR1, TNFR2, ICAM-1, VCAM-1, and MCP-1 mRNA expression as previously described.

**Statistical analysis.** Data are shown as means ± SD or medians (25th and 75th percentiles). ANOVA or an unpaired t-test was used to evaluate differences between means. Furthermore, Pearson’s correlation coefficient was estimated to evaluate the strength of the relationship between continuous outcomes of interest. P values of <0.05 were considered statistically significant.

**RESULTS**

**Biochemical parameters.** Biochemical characteristics are shown in Table 1. Before starting the experiment, we measured body weight, HbA1c, casual blood glucose, systolic blood pressure, ACR, NAG, and 8-OHdG of each animal at 8 wk of age. These levels, except for NAG and 8-OHdG in untreated KK-A^v^ mice, were already significantly higher than those in KK mice. However, these levels did not differ between untreated and ETN-treated KK-A^v^ mice. Levels of ACR in untreated KK-A^v^ mice gradually increased after 8 wk of age, whereas those in KK mice did not show any tendency to increase. Levels of ACR, HbA1c, casual blood glucose, NAG, and 8-OHdG at 16 wk of age in ETN-treated KK-A^v^ mice were significantly improved compared with those in untreated KK-A^v^ mice. However, levels of lipids (total cholesterol and triglyceride) and adiponectin did not differ between these two groups.
Fig. 6. Immunohistochemical staining of AZAN in mouse renal sections (A–C). Interstitial fibrosis is more prominent in untreated KK-Ay mice (B; magnification: ×200) than in KK mice and ETN-treated KK-Ay mice (A and C; magnification: ×200). D: bar graph of AZAN-stained areas/WIA. *P < 0.05 vs. KK mice; †P < 0.05 vs. untreated KK-Ay mice.

Fig. 7. Immunohistochemical TUNEL and cleaved caspase-3 staining in renal sections of mice at 16 wk of age. A, E, and I: KK mice; B, F, and J: untreated KK-Ay mice; C, G, and K: ETN-treated KK-Ay mice. H: positive control. Images were taken at 400-fold (A–C and E–K) magnification. The numbers of TUNEL- and cleaved caspase-3-positive cells in untreated KK-Ay mice were few and were not significantly different from ETN-treated KK-Ay mice.
monoclonal and polyclonal antibodies, respectively. In the experiments for F4/80 and 4-HNE were performed using Immunohistochemical oxidative stress molecules in the kidney. decreased after the treatment with ETN.

way, and mRNA levels of MCP-1 were also significantly VCAM-1, which are downstream molecules of the TNF pathway.

mRNA levels of ICAM-1 and /H9251 TTN-

 KK-Ay mice were significantly larger than those in KK mice, untreated KK-Ay mice, and ETN-treated KK-Ay mice. mRNA levels of ICAM-1 and VCAM-1, which are downstream molecules of the TNF pathway, and mRNA levels of MCP-1 were also significantly decreased after the treatment with ETN.

Immunohistochemical staining and mRNA levels of TNF pathway molecules in the kidney. Immunohistochemical experiments were performed using polyclonal antibodies. TNF-α-positive cells were localized in the podocyte and mainly in the tubules and interstitium. TNF-α-stained areas in untreated KK-Ay mice were significantly larger than those in KK mice, but these areas did not differ between ETN-treated and untreated KK-Ay mice (Fig. 1, A–G). In contrast to TNF-α, TNFR1 expression was not observed in the glomerular and distal tubular areas. TNFR1-positive cells were mainly localized in the proximal tubules, and those stained areas did not differ among the three groups (Fig. 1, H–N). The staining pattern of TNFR2-positive cells resembled that of TNF-α. TNFR2-stained areas in ETN-treated KK-Ay mice were significantly smaller than those in untreated KK-Ay mice (Fig. 1, O–U). As shown in Fig. 2, in the real-time PCR analysis, kidney mRNA levels of TNF-α, TNFR2, ICAM-1, VCAM-1, and MCP-1 in untreated KK-Ay mice were significantly higher than those in KK mice. mRNA levels of TNFR2 in ETN-treated KK-Ay mice were significantly lower than those in untreated KK-Ay mice. On the other hand, mRNA levels of TNF-α and TNFR1 did not differ between ETN-treated and untreated KK-Ay mice. mRNA levels of ICAM-1 and VCAM-1, which are downstream molecules of the TNF pathway, and mRNA levels of MCP-1 were also significantly decreased after the treatment with ETN.

Immunohistochemical staining of other inflammatory and oxidative stress molecules in the kidney. Immunohistochemical experiments for F4/80 and 4-HNE were performed using monoclonal and polyclonal antibodies, respectively. In the diabetic condition, the number of F4/80, the intensities of renal NF-κB activation, and 4-HNE-stained areas were increased. F4/80-positive cells were mainly localized in the peritubular capillaries and proximal tubules. The number of F4/80-positive cells in the tubulointerstitium and the intensities of renal NF-κB activation of ETN-treated KK-Ay mice were significantly decreased compared with those in untreated KK-Ay mice (Figs. 3 and 4). 4-HNE-positive cells were mainly localized in the tubules and interstitium. 4-HNE-stained areas in ETN-treated KK-Ay mice were significantly smaller than those in untreated KK-Ay mice (Fig. 5). Taken together, the renal fibrosis area, which was represented by AZAN staining in ETN-treated KK-Ay mice, was significantly improved after treatment with ETN, probably due to improvements of inflammation and oxidative stress (Fig. 6).

Involvement of apoptosis and necroptosis in the TNF-α-TNFR1 pathway. The TNF-α-TNFRI signaling pathway induces apoptosis through the activation of caspase-3 and caspase-8 (10). At the same time, recent studies have demonstrated that this pathway also can induce programmed necrosis (necroptosis) through RIPK1-RIPK3 activation under suppression of caspase activation. Cleaved caspase-3-positive cells were localized in the tubules and interstitium with double staining with 4’,6-diamidino-2-phenylindole. The number of caspase-3- and TUNEL-positive cells in untreated KK-Ay mice were few and were not significantly different from ETN-treated KK-Ay mice (Fig. 7). Protein levels of RIPK1 and RIPK3, which are essential for TNF-α-induced necroptosis (14, 26), and MLKL, which is a crucial downstream substrate of RIPK3 in the necrosis pathway (13, 47, 47a, 31a), did not differ among

![RIPK3 and MLKL expression](image-url)

Fig. 8. Immunohistochemical receptor-interacting protein kinase (RIPK)1 staining in renal sections of mice at 16 wk of age. A: KK mice; B: untreated KK-Ay mice; C: ETN-treated KK-Ay mice. Images were taken at 100-fold magnification. RIPK1 expression levels in renal tubulointerstitial areas did not differ among KK mice, untreated KK-Ay mice, and ETN-treated KK-Ay mice. D: bar graph of RIPK1-stained areas/WIA.

![Western blot analysis of RIPK3 and MLKL](image-url)

Fig. 9. Western blot analysis of RIPK3 and mixed lineage kinase domain-like (MLKL) protein in mice at 16 wk of age. RIPK3 and MLKL protein expression levels in the whole kidney did not differ among KK mice, Untreated KK-Ay mice, and ETN-treated KK-Ay mice.

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the three groups (Figs. 8 and 9). However, mRNA levels of RIPK3 and MLKL increased in the diabetic condition, and these levels did not change after treatment with ETN (Fig. 10).

Levels of serum/urinary TNFR1 and TNFR2. Levels of TNF-α in sera and urine were lower than sensitivity (data not shown). Some authors consider soluble TNFRs as a proxy for exposure to TNF-α (2). Thus, we also measured TNFRs in sera and urine. As shown in Fig. 11, A–D, untreated KK-Ay mice showed higher levels of urinary TNFR1 and serum/urinary TNFR2 compared with nondiabetic KK mice. Although serum

**Fig. 10. Renal mRNA levels of RIPK1, RIPK3, and MLKL in mice at 16 wk of age.** Renal mRNA expression levels of RIPK1 did not differ among KK mice, untreated KK-Ay mice, and ETN-treated KK-Ay mice. Renal mRNA levels of RIPK3 and MLKL in untreated KK-Ay mice were significantly higher than those in KK mice. Levels of RIPK3 and MLKL did not differ between untreated and ETN-treated KK-Ay mice. *P < 0.05 vs. KK mice.

**Fig. 11. Serum and urinary TNFRs in mice at 16 wk of age.** A–D: concentrations of urinary/serum soluble (s)TNFRs. Untreated KK-Ay mice had higher levels of urinary TNFR1 and urinary/serum TNFR2 compared with nondiabetic mice. Although serum TNFR1 levels in untreated KK-Ay mice tended to be higher compared with nondiabetic KK mice, significant differences were not observed between the two groups. Treatment with ETN partially recovered serum TNFR2 only. E–L: correlation between the albumin-to-creatinine (Cr) ratio (ACR) and urinary (E–H) and serum (I–L) TNFRs. In only untreated mice (E and F), urinary TNFR1 or TNFR2 was significantly correlated with ACR. In only ETN-treated mice (G and H), serum TNFR1 or TNFR2 was significantly correlated with ACR. In only untreated mice (I and J), serum TNFR1 or TNFR2 was significantly correlated with ACR. In only ETN-treated mice (K and L), there was no correlation between urinary sTNFR1 or sTNFR2 and ACR. M–P: correlation between urinary and serum TNFR1 or TNFR2. In only untreated mice (M and N), urinary sTNFR1 and sTNFR2 were moderately or strongly correlated with ACR, respectively. In only ETN-treated mice (O and P), there was no correlation between urinary and serum TNFR1 or TNFR2.
TNFR1 levels in untreated KK-A\(^\text{y}\) mice tended to be higher compared with nondiabetic KK mice, a significant difference was not observed between these two groups (\(P = 0.10\)). Furthermore, treatment with ETN showed little change in the concentrations of serum TNFR1 and urinary TNFRs. On the other hand, serum TNFR2 levels in KK-A\(^\text{y}\) mice were significantly decreased after treatment with ETN.

In only KK and untreated KK-A\(^\text{y}\) mice, both urinary TNFR1 (\(r = 0.86, P < 0.001\)) and TNFR2 (\(r = 0.82, P < 0.001\)) were more significantly correlated with ACR than serum TNFR1 (\(r = 0.47, P = 0.06\)) and TNFR2 (\(r = 0.67, P < 0.005\)), respectively (Fig. 11, E, F, I, J). In addition, the correlation between serum and urinary TNFRs was moderate (TNFR1: \(r = 0.64, P < 0.005\); TNFR2: \(r = 0.86, P < 0.001\); Fig. 11, M and N). Therefore, urinary TNFRs might represent local (kidney tissue) secretion rather than systemic (whole body) secretion. When the analysis was repeated only in ETN-treated mice, there was no association between serum/urinary TNFRs and ACR (Fig. 11, G, H, K, L, O, and P).

TACE activity in untreated KK-A\(^\text{y}\) mice was significantly increased compared with KK mice but did not differ between ETN-treated and untreated KK-A\(^\text{y}\) mice (Fig. 12A). Moreover, TACE activity in the kidneys was more strongly correlated with urinary TNFRs than serum TNFRs (Fig. 12, B–I). These results suggest that the diabetic condition increased TACE activity in the kidneys, which resulted in an increase of urinary TNFRs in diabetic mice. However, treatment with ETN did not affect TACE activity in the kidneys.

**In vitro experiments.** To investigate the regulation of the TNF pathway, we studied TNFR1, TNFR2, ICAM-1, VCAM-1, and MCP-1 mRNA levels in mProx cells in the absence or presence of TNF-\(\alpha\) or HG. As shown in Fig. 13, HG, but not osmotic stimulation by mannitol, markedly increased these mRNA expression levels in mProx cells. Furthermore, stimulation by HG with TNF-\(\alpha\) increased these levels more than with HG alone. Treatment with ETN partly recovered both TNFR levels, which were stimulated by TNF-\(\alpha\) but not HG. The expression patterns of ICAM-1 and VCAM-1 resembled those of TNFRs. However, the expression patterns of MCP-1 were a little bit different. Treatment with ETN recovered MCP-1 levels, which were stimulated by not only TNF-\(\alpha\) but also HG.

**DISCUSSION**

ETN did not affect the mRNA and protein levels of either TNF-\(\alpha\) or TNFR1 in the kidneys of diabetic mice. However, it ameliorated albuminuria and the mRNA levels of TNFR2, ICAM-1, and VCAM-1, the latter two of which are down-
stream molecules of the TNF pathway. Also, ETN inhibited MCP-1 mRNA levels and macrophage infiltration into the kidney. There was little apoptosis in the kidneys of diabetic mice, although further study is needed about the involvement of necroptosis because of the discrepancies between mRNA and protein levels of necroptosis-related molecules. These results suggest that ETN may exert a renal protective effect via inhibition of the inflammatory pathway activated by TNFR2 rather than TNFR1.

Consistent with our present findings, Venegas et al. (48) reported that ETN suppresses a downstream signaling of target of TNF-α, such as NF-κB in the renal cortex, although ETN did not affect the expression of membrane TNF-α in a mouse model of systemic lupus erythematosus. This lack of drug effect might be attributable to the following characteristics of ETN. First, ETN predominantly binds to circulating (soluble) TNF-α and does not affect the expression of membrane TNF-α in the kidney (48). Second, the binding of ETN (a diametric molecule) to membrane TNF-α is not as strong as that of infliximab (a trimeric molecule) due to the differences in structure, raising the possibility that ETN causes a decrease in TNF-α activity but that the effect is not strong enough to decrease membrane TNF-α expression in the kidney (44). We speculate that a continuance of lower serum TNF-α levels will result in a decrease of membrane TNF-α in the kidneys over time.

Regarding the renal mRNA and protein levels of TNFRs, a variety of results have been reported up to now. Renal TNFR1 levels decreased in a rat model with tubulointerstitial fibrosis (33). Those levels increased in a mouse model with systemic lupus erythematosus (50) but were not altered in a mouse model with membranoproliferative glomerulonephritis (33). Those levels decreased in a rat model with tubulointerstitial fibrosis (33). Those levels increased in a mouse model with systemic lupus erythematosus (50) but were not altered in a mouse model with membranoproliferative glomerulonephritis (33). Those levels decreased in a rat model with tubulointerstitial fibrosis (33). Those levels increased in a mouse model with systemic lupus erythematosus (50) but were not altered in a mouse model with membranoproliferative glomerulonephritis (33). Thus, renal TNFR1 levels vary depending on the pathological conditions. In this study, renal TNFR1 levels did not differ between mice with and without diabetes, suggesting that this receptor is not associated with the progression of DN. With
In conjunction with a previous report (19) on diabetic animals, but also hyperglycemia (42), interferon-%, and VEGF (11). In this study, renal TNF-% levels, even though it appears most likely to decrease serum TNF-% levels through not only TNF-% but also HG. LG, low glucose; Mann, mannitol. *P < 0.05.

It has been suggested that circulating TNFRs represent a buffer system that may prolong the biological actions (a slow release reservoir) of TNF-% or may function as decoys for TNF-% (1). In this study, serum and urinary TNFRs in diabetic mice were increased, as they are in subjects with diabetes (21, 34). The result that ETN did not greatly affect serum TNFR levels, even though it appears most likely to decrease serum TNF-% levels, suggests that serum TNF-% and TNFR levels might be independently regulated. Recent clinical studies (21, 40) in diabetic patients from the Joslin cohort support this idea. TACE activity is thought to partly affect circulating TNFR levels (7, 43) and elevates by not only increasing TNF-% (11) but also hyperglycemia (42), interferon-%, and VEGF (11). In conjunction with a previous report (19) on diabetic animals, TACE activity was increased in the kidneys of untreated KK-A% mice. However, ETN did not affect TACE activity. Thus, serum/urinary TNFRs levels might have been controlled by other factors in addition to TACE. In fact, on top of the 28-kDa ectodomain product of TNFR1 of proteolytic cleavage, which is activated by a metalloprotease such as TACE, TNFR1 in human sera and lung epithelial lining fluid has been recently reported to be released in the intact form through exocytosis (full-length 55-kDa protein) and to circulate afterward in exosome-like vesicles (25).

Treatment with ETN improved not only albuminuria but also glycemic control, such as HbA1c and blood glucose levels. These improvements might be caused by ETN treatment, which decreased levels of TNF-%. TNF-% plays an important role in glycemic control by an increase in insulin resistance or a decrease in adiponectin (19). In this regard, Lacerda et al. (30) demonstrated that TNF knockout mice with streptozotocin-induced diabetes have a favorable effect on glycemic control. Furthermore, glycemic control improved after the administration of ETN in rheumatoid arthritis patients with concomitant diabetes (22). Thus, we cannot exclude the possibility that ETN might have ameliorated nephropathy through the improvement of glycemic control, although ETN did not alter adiponectin levels. In vitro, ETN partly recovers both TNFR mRNA expressions, which were stimulated by TNF% but not HG, suggesting that ETN might ameliorate DN by inhibiting the TNFα-TNFR pathway rather than HG stimula-
tion. Different from animal experiments, stimulation by HG and/or TNF-α increased not only TNFR2 but also TNFR1 mRNA expression in mProx cells. This discrepancy may be caused by differences of method between in vitro and in vivo, the intensity of TNF-α concentrations used in this study or the presence of other factors that might increase TNFR expression (49).

In conclusion, it appears that ETN improved the progression of DN through inhibition of the predominantly anti-inflammatory action of the TNF-α-TNFR2 pathway.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


