Blockade of Death Ligand TRAIL Inhibits Renal Ischemia Reperfusion Injury

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Renal ischemia-reperfusion injury (IRI) is a leading cause of acute kidney injury (AKI). Many investigators have reported that cell death via apoptosis significantly contributed to the pathophysiology of renal IRI. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily, and induces apoptosis and inflammation. However, the role of TRAIL in renal IRI is unclear. Here, we investigated whether TRAIL contributes to renal IRI and whether TRAIL blockade could attenuate renal IRI. AKI was induced by unilateral clamping of the renal pedicle for 60 min in male FVB/N mice. We found that the expression of TRAIL and its receptors were highly upregulated in renal tubular cells in renal IRI. Neutralizing anti-TRAIL antibody or its control IgG was given 24 hr before ischemia and a half-dose booster injection was administered into the peritoneal cavity immediately after reperfusion. We found that TRAIL blockade inhibited tubular apoptosis and reduced the accumulation of neutrophils and macrophages. Furthermore, TRAIL blockade attenuated renal fibrosis and atrophy after IRI. In conclusion, our study suggests that TRAIL is a critical pathogenic factor in renal IRI, and that TRAIL could be a new therapeutic target for the prevention of renal IRI.

Key words: TRAIL, apoptosis, TNF superfamily, ischemia reperfusion injury, fibrosis

I. Introduction

Acute kidney injury (AKI) remains a significant clinical problem because of high mortality and morbidity rates [44]. Moreover, recent studies have reported that a significant proportion of patients with AKI develop a predisposition toward chronic kidney disease (CKD) [5, 6]. However, there is no available therapy that can improve not only the mortality rate of AKI patients but also renal prognosis.

Renal ischemia reperfusion injury (IRI) is a leading cause of AKI in both allografts and native kidneys [28]. The pathophysiology of renal IRI is complicated by the fact that endothelial cell damage, tubular necrosis, tubular apoptosis [25], inflammation and tubular cell proliferation occur simultaneously [3]. Many investigators have reported that cell death via apoptosis significantly contributed to the pathophysiology of renal IRI [2]. Particularly, members of the tumor necrosis factor (TNF) superfamily have been implicated in the pathogenesis of renal IRI. TNFα (TNFSF1A) and Fas ligand (FasL, TNFSF6) have been reported as being upregulated in renal tubular cells in renal IRI, and the blockade or deficiency of these molecules protected mice from renal IRI [10, 17]. It was recently reported that blockade of TNF-like weak inducer of apoptosis (TWEAK, TNFSF12) or its receptor, fibroblast growth factor-inducible 14 (Fn14), prevented renal IRI and

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subsequent renal fibrosis [19]. Thus, the TNF superfamily members play crucial roles in the pathogenesis of renal IRI.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), also known as Apo2L (or officially as TNFSF10), is a member of the TNF superfamily [42]. TRAIL is expressed in a variety of tissues [42], and plays an important role in regulating many biological processes including apoptosis, cell survival, the immune response and inflammation [4, 18, 26, 34]. TRAIL interacts with three membrane-bound receptors and one soluble receptor in mice. DR5 (Tnfrsf10b, TRAIL-R2) has a cytoplasmic death domain and can promote the activation of Caspase-3 through Caspase-8 [43]. Two other membrane-bound receptors, mDcTRAILR1 (Tnfrsf23) and mDcTRAILR2 (Tnfrsf22) and a soluble receptor, osteoprotegerin (Tnfrsf11b, OPG), may function as decoy receptors and may be involved in non-apoptotic signaling [14, 30]. TRAIL does not induce apoptosis under normal physiological conditions [8, 46] but does induce cellular apoptosis during inflammation [46]. Because of this specificity and selectivity, TRAIL and its receptors could be a novel therapeutic target for curing diseases, without damaging normal tissue.

Oncological research conducted with mice has reported that recombinant TRAIL and anti-DR5 agonistic antibodies are substantially tumoricidal, and display no apparent toxicity [1, 20]. Therefore, some researchers have begun using them as a therapeutic agent against human cancers [39]. In nephrology research, TRAIL has been proposed as a novel therapeutic target for human diabetic nephropathy [15, 24]. However, it remains unknown whether TRAIL contributes to renal AKI.

In this study, we found that the expression of TRAIL and its receptors were upregulated in renal IRI, which correlated with apoptosis of renal tubular cells. We then investigated what contribution endogenous TRAIL had in renal IRI and investigated the therapeutic potential of TRAIL blockade in renal IRI.

II.   Materials and Methods

Animal treatments

Experiments were performed using male FVB/N mice (6–8 weeks old, 22–26 g). Animals were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium. AKI was induced by unilateral clamping of the renal pedicle with arterial microclamps. We determined the critical point of ischemic duration at which the kidney falls into renal atrophy (unpublished observations). An ischemic duration of 60 min or more would almost certainly cause renal atrophy, whereas a duration of less than 60 min would allow the kidney function to be restored. To observe renal atrophy after IRI, we set the duration of ischemia for 60 min. The left renal pedicle was occluded by clamping the left renal artery for 60 min, followed by reperfusion. Sham surgery consisted of an identical procedure with the exception of the application of arterial microclamps. All operated

mice were placed on a homeothermic table to maintain body temperature at 40°C until emergence.

Because anti-TRAIL monoclonal antibody (N2B2) specifically binds to mouse TRAIL and blocks its cytotoxic activity, the antibody is used as a neutralizing antibody to mouse TRAIL [22]. The N2B2 antibody or control IgG (Sigma-Aldrich Japan, Tokyo, Japan) were injected intraperitoneally (500 μg per mouse). Antibodies were given 24 hr before ischemia and a half-dose booster injection was administered into the peritoneal cavity immediately after reperfusion, as described previously [23]. Left kidneys were collected and weighed at the indicated times after reperfusion. Experiments were performed in accordance with the Animal Care Committee regulations of Kyoto Prefectural University of Medicine.

Histological analysis

For histological examination, kidneys were fixed with 4% buffered paraformaldehyde, embedded in paraffin, and sectioned longitudinally at the center of the kidney. Kidneys were sectioned at a thickness of 4 μm. Sections were stained with periodic acid-Schiff (PAS) (Sigma-Aldrich Japan) and Masson trichrome (Applied Medical Research Laboratory, Osaka, Japan). Sections were also performed with immunohistochemistry. For all histological analyses, a minimum of three kidneys was examined and at least 10 sections per kidney were examined.

The amount of glomeruli was counted as the nephron number. Whole glomeruli were counted in a PAS-stained section containing cortex and medulla.

The degree of renal injury was evaluated in a blinded fashion using PAS-stained sections. For semi-quantitative analysis, the extent of injury tubules (determined by cell lysis, loss of brush border, and cell detachment) were scored as follows: 0: 0–25%; 1: 25–50%; 2: 50–75%; 3: 75–100%. We called this score the "Kidney Injury Score". This score was determined using 10 randomly selected fields per section.

To detect apoptotic cells, the TUNEL method was employed using an *in situ* Apoptosis Detection Kit (Takara Bio Inc., Otsu, Japan) according to the manufacture's protocol. TUNEL-positive cells were counted in all fields in a section.

For immunohistochemical analysis, sections were incubated with anti-TRAIL (Abcam Ltd., Cambridge, UK), anti-DR5 (R&D Systems Inc., Minneapolis, MN), anti-myeloperoxidase (MPO; Thermo Fisher Scientific Inc., Waltham, MA) and anti-F4/80 antibody (AbD Serotec Ltd., Oxford, UK). This was followed by standard ABC immunostaining using Vectastain ABC Elite Kit protocol (Vector laboratories, Burlingame, CA). Quantitative analyses of neutrophil and macrophage infiltration were performed by counting MPO or F4/80 positive cells in 10 randomly selected fields per section.

The fibrosis index was determined as the percentage of the aniline blue-stained area after Masson trichrome staining. Fifty consecutive non-overlapping fields of the

renal cortex and the medulla in each kidney were observed. A standard point-counting method was used to quantify the collagen fractional volume of Masson trichrome-stained sections, as described previously [38].

Western blot analysis

Proteins from homogenized total renal tissue were analyzed by western blotting using antibodies against Caspase-8 (Santa Cruz Biotechnology, Inc., CA) and actin (Cell Signaling Biotechnology, Beverly, MA) as described previously [37]. Three kidneys were used for one experiment group, and data were collected by three independent experiments.

Semi-quantitative RT-PCR and real-time quantitative RT-PCR

cDNA from kidneys and cultured cells were subject to semi-quantitative RT-PCR using the Applied Biosystems GeneAmp PCR Systems 9700, and quantitative RT-PCR using the Applied Biosystems 7300 real-time PCR system. The mRNA level of *gapdh*, *TRAIL*, *DR5*, *DcTrailR1*, *DcTrailR2*, and *OPG* were determined by RT-PCR using the following specific PCR primers: *Glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) F: caatgtgtccgtcgtggatct, R: ttgaagtcgcaggagacaacc, *TRAIL* F: gaaaagcagctaagtactcct, R: ggattcaatcttctggcctaa, *DR5* F: ccagtacctgtcagaaggga, R: ttgcatcgggtttctacgac, *DcTrailR1* F: aggaatgcaactccacagctaac, R: ttgcctccatggtttctcttcac, *DcTrailR2* F: cccatactcaaggacaatgtgag, R: gcacgattctggaaatttttggg, *OPG* F: cagctcacaagagcaaaccttcca, R: acgctgctttcacagaggtcaa. For semi-quantitative RT-PCR, expression plasmids (0.5 µg) for *DR5*, *mDcTRAILR1*, *mDcTRAILR2*, and *OPG* were used as positive controls. For quantitative PCR, *Gapdh* was used as the control gene for normalization. Three kidneys were used for one experiment group, and data were collected by three independent experiments.

Statistics

Results are expressed as means±standard error (SE) of at least three mice in each experimental group. Comparisons between two groups were performed using an unpaired *t*-test. Multiple group comparisons were performed using one-way ANOVA followed by Tukey-Kramer's post-test comparisons using Excel 2007 (Microsoft, Redmond, WA) and Statcel2 plug-in software (OMS Publishing, Saitama, Japan).

III. Results

Activation of the TRAIL signaling pathway in IRI kidneys

Renal IRI induced marked increases in the expression of *TRAIL* mRNA levels at 1 day after IRI when compared with the expression levels in sham-operated mice (Fig. 1A). In immunohistochemical analysis, the TRAIL signal in the sham kidney group displayed a low level of expression in a small number of tubules and was negative in glomeruli and interstitial tissue (Fig. 1B). In contrast, the TRAIL signal in the IRI kidney group was highly expressed in tubular epithelial cells, but glomeruli and interstitial tissues remained negative.

Expression of the following receptors mRNA that bind to TRAIL in mice were examined: *DR5*, *mDcTRAILR1*, *mDcTRAILR2*, and *OPG*. The results indicated that all of these receptors were expressed in the sham and IRI kidneys, and in the cultured wild-type mouse tubular epithelial cell line (clone Dai1) (Fig. 1C) [35]. The expression levels of *DR5* and other receptors' mRNA at 1 day after IRI were significantly increased in mice exposed to renal IRI when compared with expression levels in sham mice (Fig. 1D).

TRAIL triggers apoptosis following binding to death receptor DR5. DR5 protein was expressed in renal tubular cells at 1 day after IRI (Fig. 1E). DR5 induces apoptosis by activating Caspase-8 cleavage. Cleaved Caspase-8 showed increased levels in the IRI kidney when compared with the sham kidney (Fig. 1F).

TRAIL blockade prevented renal IRI and reduced tubular apoptosis

As the TRAIL signal was activated in renal IRI, we sought to treat IRI kidneys with a TRAIL neutralizing antibody. Since anti-TRAIL monoclonal antibody (N2B2) specifically binds to mouse TRAIL and blocks its cytotoxic activity, the antibody is used as a neutralizing antibody to mouse TRAIL [22]. First, we examined whether TRAIL blockade suppressed tubular injury. In our IRI system, the tubular injury peak was observed at 3 days after renal IRI (data not shown). Tubular injury was significantly repressed in kidneys treated with anti-TRAIL antibody compared with the corresponding control IgG-treated group at 3 days after renal IRI (Fig. 2A). The kidney injury score was significantly decreased by the blockade of TRAIL at 3 days after renal IRI (Fig. 2B).

We also evaluated apoptosis of renal tubular cells caused by renal IRI by TUNEL assay. The number of apoptotic cells in kidneys treated with anti-TRAIL antibody was significantly lower than those of the control IgG-treated kidney at 1 day after renal IRI (Fig. 3A and B). Moreover, we examined whether TRAIL blockade suppressed the activation of Caspase-8 in the IRI kidney. As shown in Figure 3C, cleaved Caspase-8 was significantly reduced by blockade of TRAIL at 1 day after renal IRI (Fig. 3C).

TRAIL blockade inhibits pro-inflammatory cytokine and chemokine production in the IRI kidneys

We examined whether TRAIL blockade suppressed local inflammation. The mRNA expression of pro-inflammatory cytokines and chemokines in the IRI kidney were examined by quantitative PCR analysis. The expression levels of several pro-inflammatory cytokines including *TNFα*, *FasL* and *interleukin-1β (IL-1β)*, and several chemokines such as *monocyte chemotactic protein-1* (*MCP-1*) and *macrophage inflammatory proteins-2* (*MIP-2*), were significantly downregulated by TRAIL blockade (Fig. 4A).

### TRAIL blockade inhibits neutrophil and macrophage infiltration in the IRI kidney

We directly examined neutrophil accumulation in the IRI kidney by MPO staining. A considerable number of neutrophils were identified in the control IgG-treated kidneys at 1 day after renal IRI (Fig. 4B). Using quantitative analysis, we found a dramatic reduction in the number of accumulated neutrophils in kidneys treated with anti-TRAIL antibody (Fig. 4C).

We also examined macrophage accumulation by F4/80
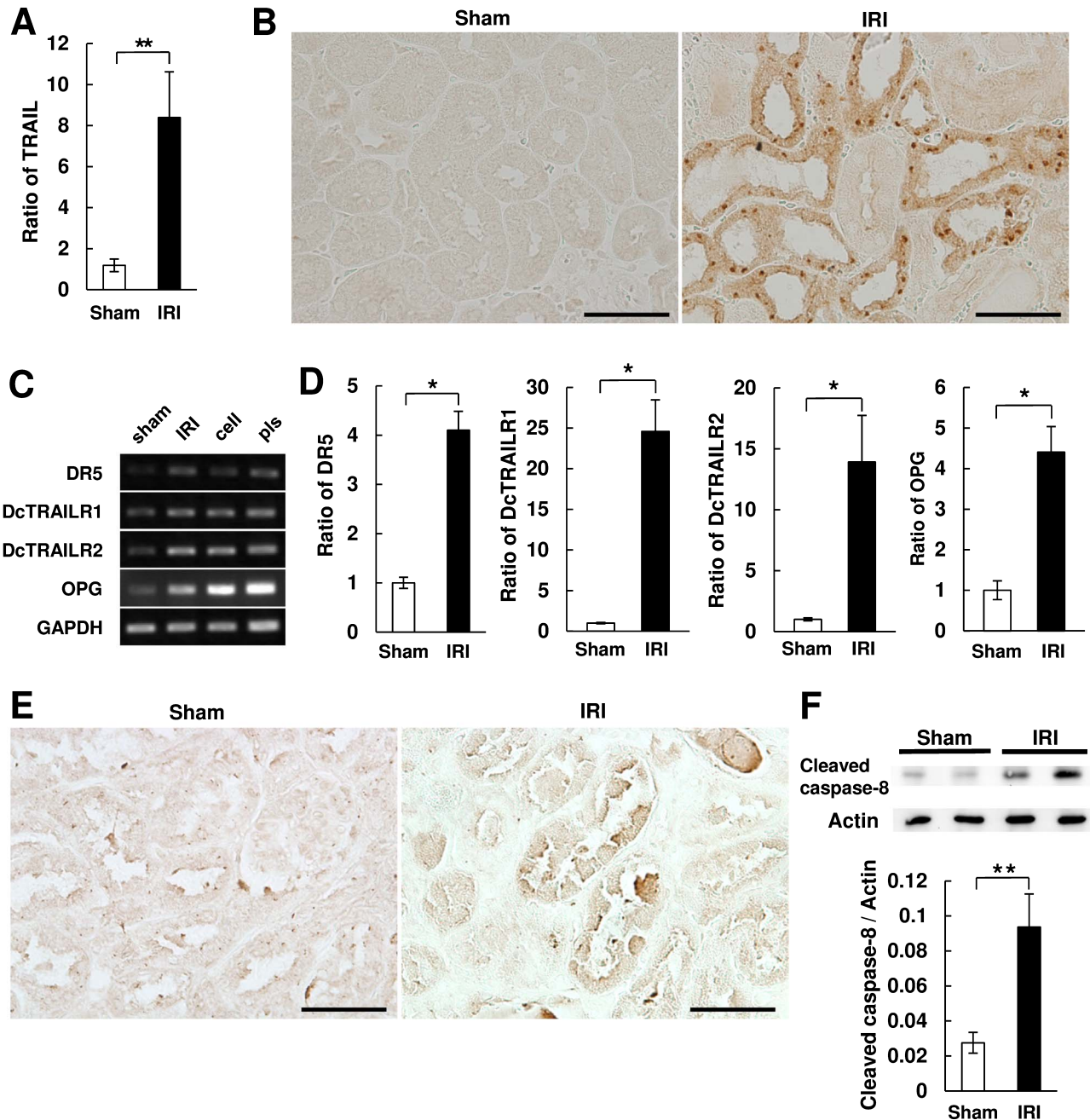
Fig. 1. Activation of TRAIL signaling pathway in renal IRI. (**A**) Expression change of *TRAIL* mRNA in the kidney at 1 day after IRI. (**B**) Immunohistochemical staining of TRAIL-expressing cells in the kidney at 1 day after IRI. Bar=50 μm. (**C**) Expression of *DR5, mDcTRAILR1, mDcTRAILR2,* and *OPG* mRNA in the sham and IRI kidneys and a renal tubular cell line. Expression plasmids for *DR5, mDcTRAILR1, mDcTRAILR2,* and *OPG* were used as positive controls. (**D**) Quantitative analysis of *DR5, mDcTRAILR1, mDcTRAILR2,* and *OPG* mRNA expression in the kidney at 1 day after IRI. (**E**) Immunohistochemical staining of DR5 expressed cells in the kidney at 1 day after IRI. Bar=50 μm. (**F**) Caspase-8 activation in the kidney at 1 day after IRI. $*P<0.05$, $**P<0.01$ vs. sham.
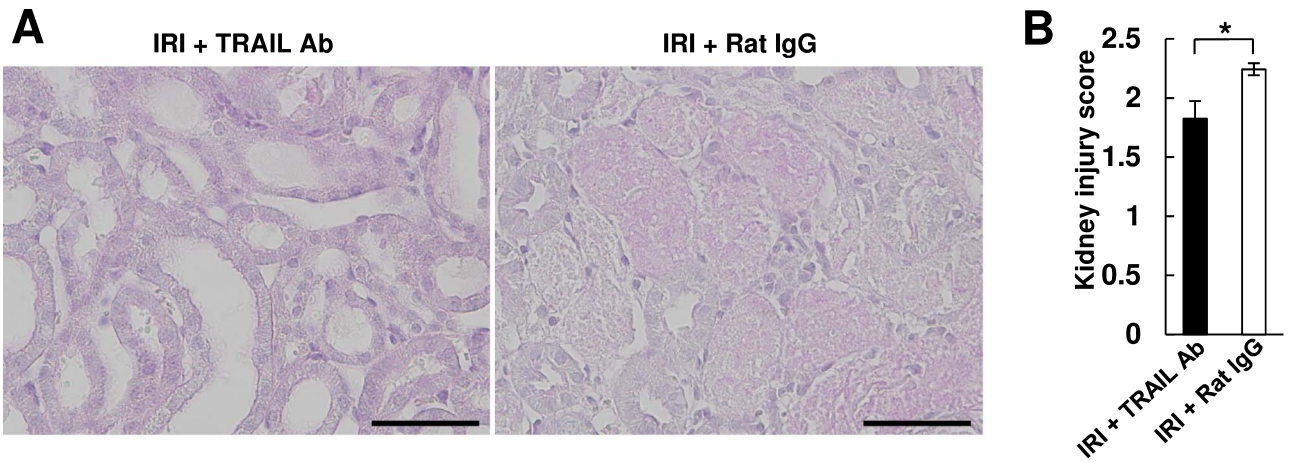
Fig. 2. Inhibition of renal injury by TRAIL blockade in the IRI kidney. (**A**) PAS-stained sections of the treated kidney at 3 days after IRI. Bar=50 μm. (**B**) Kidney injury score at 3 days after IRI. IRI+TRAIL Ab: group treated with anti-TRAIL antibody; IRI+Rat IgG: group treated with Rat control IgG. *$P<0.05$.
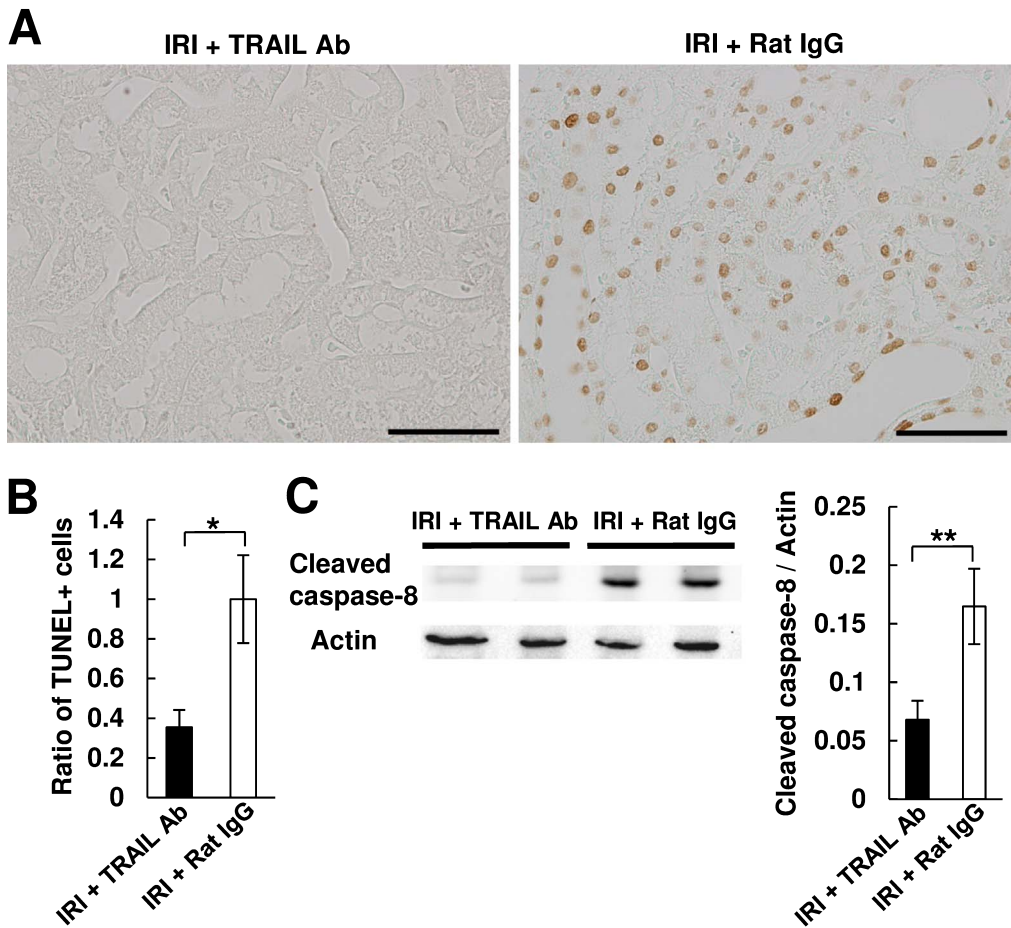
Fig. 3. Inhibition of tubular apoptosis by TRAIL blockade in the IRI kidney. (**A**) TUNEL-stained sections of the treated kidney at 1 day after IRI. Bar=50 μm. (**B**) Number of TUNEL positive cells in antibody-treated kidneys at 1 day after IRI. (**C**) Caspase-8 activation at 1 day after IRI. IRI+TRAIL Ab: group treated with anti-TRAIL antibody; IRI+Rat IgG: group treated with Rat control IgG. *$P<0.05$. **$P<0.01$.
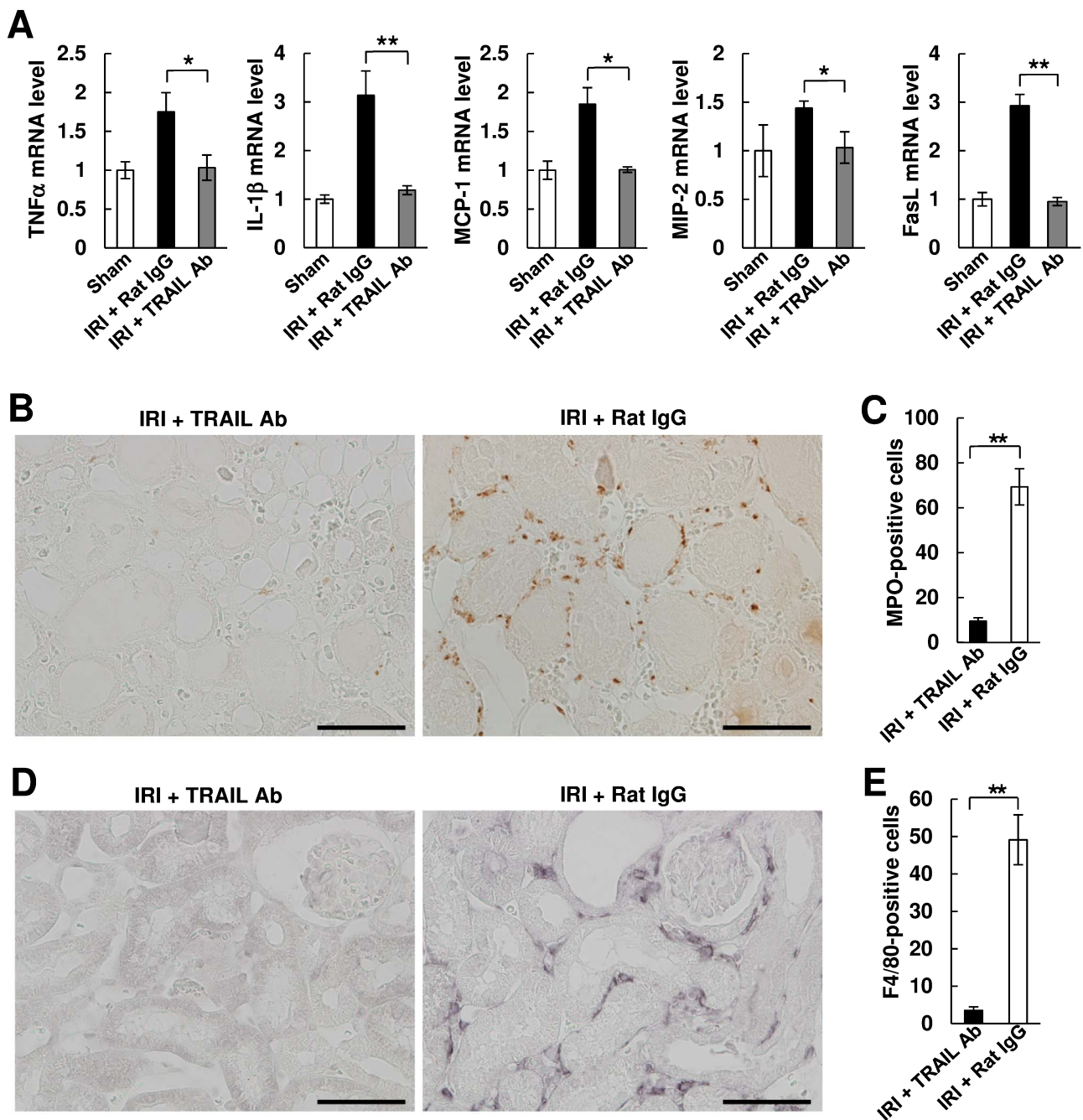
Fig. 4. Inhibition of pro-inflammatory cytokine/chemokine production and neutrophil/macrophage infiltration by TRAIL blockade in the IRI kidney. (**A**) Quantitative analysis of *TNFα, interleukin-1β* (*IL-1β*), *monocyte chemotactic protein* (*MCP-1*), *macrophage inflammatory protein-2* (*MIP-2*) and *FasL* mRNA expression by real-time RT-PCR at 1 day after IRI. (**B**) Detection of neutrophils by MPO staining in the kidney at 1 day after IRI. Bar=50 μm. (**C**) Quantitative analysis of MPO-positive cells. (**D**) Detection of macrophages by F4/80 staining at 3 days after IRI. Bar=50 μm. (**E**) Quantitative analysis of F4/80 positive cells. IRI+TRAIL Ab: group treated with anti-TRAIL antibody; IRI+Rat IgG: group treated with Rat control IgG. *$P<0.05$. **$P<0.01$.

staining. Interstitial macrophages accumulated in the control IgG-treated kidneys at 3 days after renal IRI. Fewer interstitial macrophages were detected in kidneys treated with anti-TRAIL antibody (Fig. 4D), with the differences observed being statistically significant (Fig. 4E).

TRAIL blockade prevented renal atrophy and chronic fibrosis induced by IRI

As kidneys exhibited interstitial fibrosis and renal atrophy at 28 days after renal IRI, we examined whether TRAIL blockade could improve renal atrophy and inter-
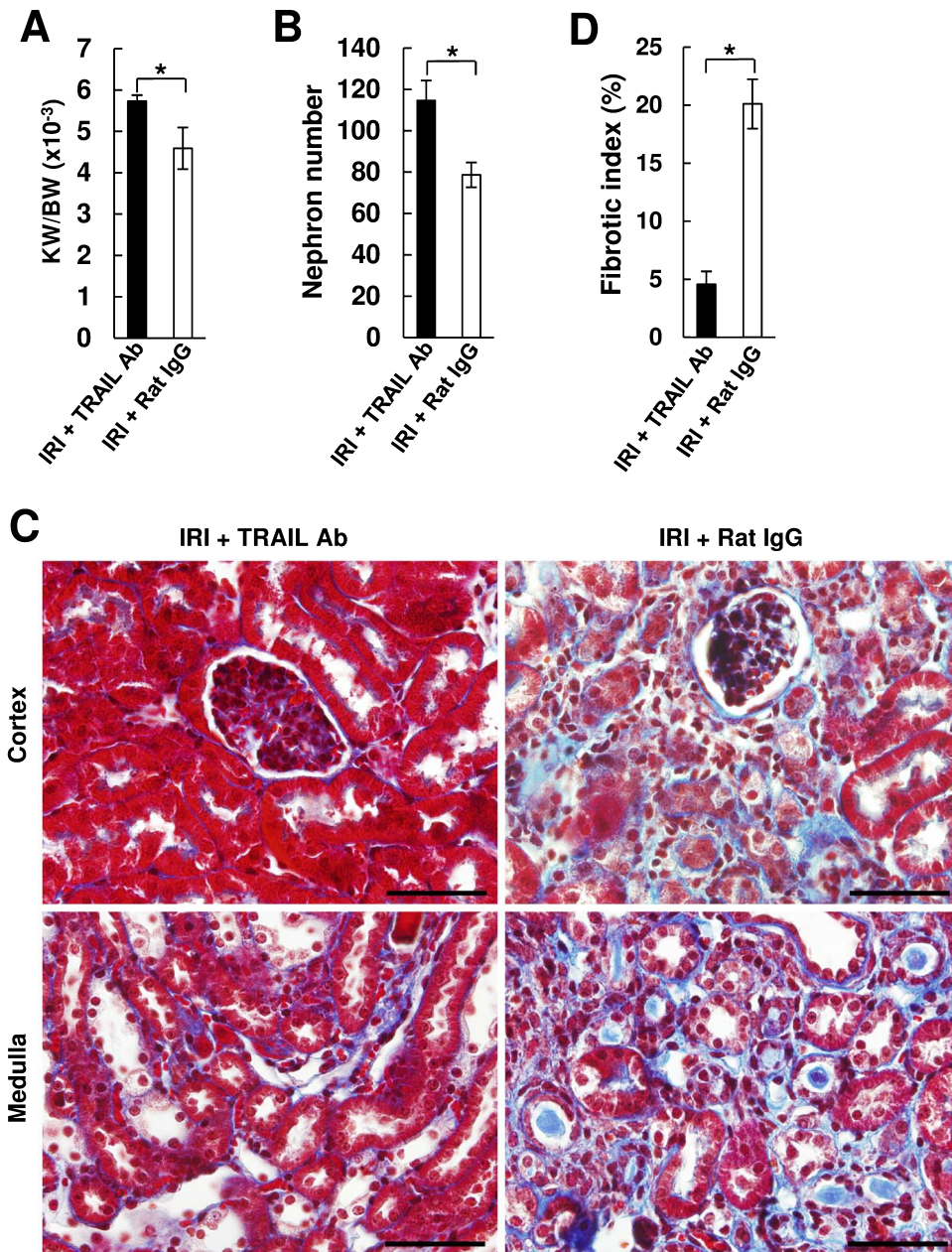
Fig. 5. Inhibition of renal atrophy by TRAIL blockade in the IRI kidney. (**A**) Kidney weight/body weight (KW/DW) of treated kidneys at 28 days after IRI. (**B**) Nephron number observed in treated kidneys at 28 days after IRI. (**C**) Masson trichrome stained sections of treated kidneys at 28 days after IRI. Bar=50 μm. (**D**) Fibrosis index of treated kidneys at 28 days after IRI. IRI+TRAIL Ab: group treated with anti-TRAIL antibody; IRI+Rat IgG: group treated with Rat control IgG. *P<0.05.

stitial fibrosis. We observed that kidney weight/body weight (KW/BW) significantly improved with treatment with anti-TRAIL antibody when compared with the control IgG (Fig. 5A). The loss of nephrons was prevented by TRAIL blockade (Fig. 5B). Renal interstitial fibrosis significantly improved (Fig. 5C), and the fibrosis index significantly decreased with TRAIL blockade (Fig. 5D).

IV. Discussion

TRAIL and DR5 are expressed in some renal distal tubules in the normal kidney [26, 36]. Expression of *mDcTRAIL-R1* was not detected in the normal kidney [42], and expression of *mDcTRAIL-R2* was not reported in normal kidney. In our study, we observed that both TRAIL and DR5 were upregulated in tubular epithelial cells after renal IRI, and that other receptors were also upregulated in

the IRI kidney. We also observed that Caspase-8, downstream of TRAIL signal, was activated in renal IRI. These results suggested that the TRAIL signal was activated in the IRI kidney.

The pathophysiology of renal IRI is complicated including tubular injury, tubular apoptosis and inflammation. Previous research has indicated that TRAIL is a multifunctional cytokine, acting as an apoptosis inducer, a pro-survival factor and an inflammatory mediator [7, 11, 26, 33], which is consistent with other TNF superfamily cytokines. Further, TRAIL can exert different functions that also include proliferation, migration, and maturation depending on the cellular system. For example, TRAIL has been shown to promote cell proliferation on endothelial and vascular smooth muscle cells and to regulate erythroid and monocytic maturation [31–33]. Our results showed that TRAIL promoted injury and apoptosis in the tubular cells of IRI kidney. In particular, TRAIL could directly promote tubular apoptosis in renal IRI because our result also showed that TRAIL blockade repressed Caspase-8. On the other hand, it is known that FasL/Fas signaling pathway participates in tubular injury and apoptosis in renal IRI [23]. Our results showed that TRAIL blockade represses FasL mRNA expression. We suggested that TRAIL was located upstream of FasL/Fas signaling pathway and that TRAIL blockade could indirectly inhibit tubular apoptosis by repressing the activation of the FasL/Fas signaling pathway. Therefore, the anti-apoptotic function of TRAIL neutralizing antibody is likely a key mechanism improving renal IRI.

Our data also suggest that TRAIL blockade repressed the expression of pro-inflammatory cytokines and chemokines, and also resulted in the suppression of the secondary response including the infiltration of neutrophils and macrophages/monocytes in the IRI kidney. It has previously been reported that neutrophils and macrophages contribute to renal IRI [21, 27]. Therefore, the anti-inflammatory function of TRAIL neutralizing antibody may be a key mechanism for improving renal IRI. These results suggest that TRAIL blockade may exhibit its substantial therapeutic effect on renal IRI through anti-apoptotic and anti-inflammatory functions.

The exact target cells of TRAIL are unclear. Immune cells express both TRAIL and DR5, and immune cells themselves are thought to be a target of TRAIL [40]. However, our immunohistochemical analysis did not clearly exhibit TRAIL and DR5 expression on infiltrating cells in the IRI kidney. Our results showed TRAIL and DR5 were only upregulated in renal tubular cells. Furthermore, TRAIL induces apoptosis of cultured renal tubular cells directly [26]. We therefore hypothesize that TRAIL is secreted from renal tubular cells and may stimulate neighboring tubular cells through DR5 in a paracrine manner. It was expected that the tubular cells that received TRAIL-DR5 signal were induced apoptosis through caspase, and produced several pro-inflammatory cytokines such as TNFα and IL-1β. TRAIL binds other receptors, mDcTRAIL-R1,

mDcTRAIL-R2, and OPG. It was reported that these receptors were functioning in cell survival against TRAIL-induced apoptosis [11, 29]. Although we were not able to identify the cells that the other three receptors expressed, the expression levels of these receptors were increased in the IRI kidney. We therefore speculated that these receptors are expressed in viable tubular cells in IRI kidney and protected against TRAIL-induced apoptosis. It was reported that cell cycle-arrested cells exhibit increased sensitivity towards TRAIL-induced apoptosis [13]. It was also reported that epithelial cell arrest in G2/M was observed in severe or sustained kidney injury [45]. Therefore, the role of TRAIL-induced apoptosis through DR5 might be due to the removal of the arrested cells, while the other three receptors might be concerned with the selection of the viable cells in the IRI kidney.

Recent studies have reported persistent and irreversible structural and functional changes in the post-ischemic kidney, and some reports suggest that renal post-inflammatory scarring caused by IRI might be an important contributor to the development of CKD including renal fibrosis and atrophy [16, 41]. Of the members of the TNF superfamily, only TWEAK blockade has been reported as improving renal fibrosis and viability until 30 days after renal IRI [19]. We found that TRAIL blockade was highly effective in improving renal fibrosis and atrophy until 28 days after renal IRI. This suggests that TRAIL blockade could be a new therapeutic approach against disease progression from AKI to CKD. However, TRAIL may play a role in curtailing immune responses to avoid an excessive reaction to self-antigens or infectious agents. TRAIL blockade has also been reported to exacerbate murine experimental autoimmune diseases [9, 18]. Clearance of murine cytomegalovirus was augmented in DR5-knockout mice [12]. These results suggest that the function of TRAIL depends on a fine balance between apoptotic, pro-inflammatory and immunosuppressive functions. Further research is needed to distinguish the various functions of TRAIL before a clinical application of TRAIL blockade could be developed for AKI.

In conclusion, we suggest that TRAIL plays a critical role in renal IRI, and that TRAIL blockade ameliorates renal IRI. Our findings are of clinical relevance and may provide new insights into the pathogenesis of renal IRI. This study also suggests that targeting TRAIL may be a novel therapeutic approach to overcome renal IRI.

V.   Acknowledgment

VI.   Disclosures

None.

VII. References

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