Cytokine cooperation in renal tubular cell injury: The role of TWEAK

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Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK, TNFSF12) is a member of the TNF superfamily. TWEAK activates the Fn14 receptor, and may regulate apoptosis, proliferation, and inflammation, processes that play a significant role in pathological conditions. However, there is little information on the function and regulation of this system in the kidney. Therefore, TWEAK and Fn14 expression were studied in cultured murine tubular epithelial MCT cells and in mice *in vivo*. The effect of TWEAK on cell death was determined. We found that TWEAK and Fn14 expression was increased in experimental acute renal failure induced by folic acid. Cultured tubular cells express both TWEAK and the Fn14 receptor. TWEAK did not induce cell death in non-stimulated tubular cells. However, in cells costimulated with TNFα/interferon-gamma, TWEAK induced apoptosis through the activation of the Fn14 receptor. Apoptosis was associated with activation of caspase-8, caspase-9, and caspase-3, Bid cleavage, and evidence of mitochondrial injury. There was no evidence of endoplasmic reticulum stress. A pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp prevented TWEAK-induced apoptosis, but it sensitized cells to necrosis via generation of reactive oxygen species. In conclusion, cooperation between inflammatory cytokines results in tubular cell death. TWEAK and Fn14 may play a role in renal tubular cell injury.

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Tubular cells compose most of the mass of the functioning kidney. Loss of renal tubular cells characterizes both acute and chronic renal failure.[1] Acute tubular necrosis is the most common form of acute renal failure.[2] In humans, tubular cell death was the best histopathological correlate of renal dysfunction in acute tubular necrosis.[3,4] The term acute tubular necrosis was originated in necropsy studies before the phenomenon of apoptosis had been acknowledged as a separate form of cell death. However, in human acute tubular necrosis apoptosis was the most common form of tubular cell death, albeit necrosis was also present.[3,4] Thus, the term acute tubular injury is now more widely employed and will be used throughout the text Apoptosis is an active mode of cell death under molecular control.[1] Extracellular and intracellular molecules that provide multiple regulatory and contra-regulatory pathways tightly regulate apoptosis. These molecules are potential targets for therapeutic intervention. However, the design of appropriate therapeutic strategies requires a correct understanding of the molecular regulation of renal cell apoptosis. In this regard, the role of apoptosis regulatory molecules may vary in a cell type- and stimulus-specific manner.[5] Members of the tumor necrosis factor (TNF) superfamily of cytokines regulate several cell responses, including proliferation, differentiation, and death.[6,7] Two of these cytokines, TNFα and FasL, have been extensively studied in renal injury and shown to be involved in renal damage.[8–14] TNF-like weak inducer of apoptosis (TWEAK, TNFSF12) is a recently described member of the TNF superfamily of structurally related cytokines.[15,16] TWEAK functions as a both type II transmembrane protein and as a cleaved soluble molecule. TWEAK may promote cell death, but it also modulates cell proliferation, inflammation, and angiogenesis.[15,17–20] Fibroblast growth factor-inducible 14 (Fn14) is a cell membrane TWEAK receptor.[20–22] Both TWEAK and its receptor, Fn14, are present in the healthy adult kidney.[15,21] However, there is little information on the regulation of the expression and the role of TWEAK and its receptor in kidney disease.

We now report that TWEAK induces tubular cell apoptosis in the presence of proinflammatory cytokines and that these findings may be relevant to the pathogenesis of acute renal failure.

RESULTS

Increased expression of TWEAK and Fn14 in experimental acute renal failure

In an experimental model of acute tubular injury characterized by tubular cell apoptosis,[10] expression of both TWEAK (Figure 1a and b) and its receptor (Figure 1c and d) increased both at the messenger RNA (mRNA) and protein level. Fn14 was localized to injured tubules (Figure 1e). Based on this observation we studied the contribution of TWEAK to cultured tubular cell death.

Fn14 is expressed and inducible in renal tubular cells

Renal tubular murine cortical tubular cell line (MCT) cells constitutively express TWEAK and Fn14 (Figure 2a–c). Constitutive Fn14 expression is low, but it was upregulated by proinflammatory cytokines (TNFα, interferon-gamma (INFγ)) alone or in combination (Figure 2c). Increased Fn14 expression was also observed when the ligand, TWEAK, was combined with TNFα/INFγ. This combination increased Fn14 in a time-dependent manner at the mRNA (Figure 2b) and protein (Figure 2c) levels. Quantitative polymerase chain reaction (PCR) confirmed a fourfold increase of Fn14 mRNA at 3 h, with return to baseline by 24 h (data not shown).

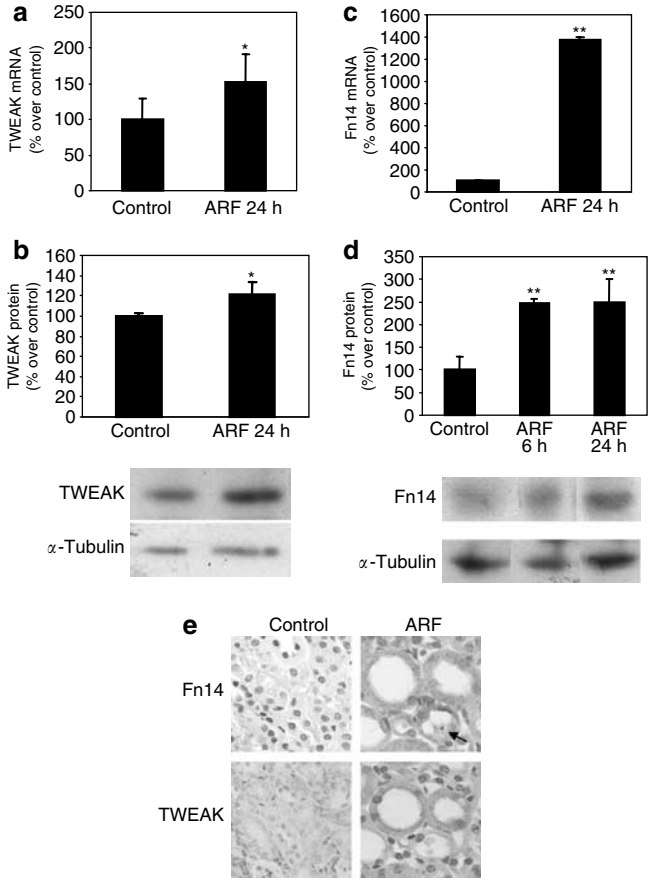

Figure 1 | Increased expression of TWEAK and Fn14 in experimental acute renal failure (ARF). In ARF induced by a single injection of folic acid the expression of (**a**) TWEAK mRNA (real-time PCR) and (**b**) protein (Western blot), (**c**) Fn14 mRNA (real-time PCR), and (**d**) protein (Western blot) were increased. (**e**) Fn14 was localized to injured, dilated tubules (arrows) (immunohistochemistry). Both proximal and distal tubules expressed Fn14, which colocalized with TWEAK mainly in proximal tubules (inset, **e**). Note the presence of apoptotic cells in the tubular lumen (arrowhead). Mean (s.d.) of five mice. $*P<0.05$ vs control, $**P<0.01$ vs control (original magnification $\times 400$).

TWEAK induces apoptosis in tubular cells exposed to proinflammatory cytokines

We next evaluated a potential lethal activity of TWEAK in tubular cells. TWEAK did not induce apoptosis in non-stimulated tubular cells (Figure 3a). By contrast, costimulation with TNFα and INFγ resulted in sensitization to TWEAK-induced apoptosis (Figure 3b). Neither INFγ nor TNFα sensitized, by themselves, to death induced by TWEAK (Figure 3c). Features of apoptosis included the presence of hypodiploid cells and morphology (nuclear shrinkage, condensation, and fragmentation as well as decreased cell size) (Figure 4a and b).

Apoptosis increased with concentration of TWEAK (Figure 3b). For further experiments we chose a concentration of 100 ng/ml of TWEAK, which induces around 30% of the cells to undergo apoptosis in 24 h (Figure 3b). Under these conditions, TWEAK-induced apoptosis was time-dependent and was apparent following 18 h of incubation (Figure 5). TNFα/INFγ induced delayed apoptosis in tubular

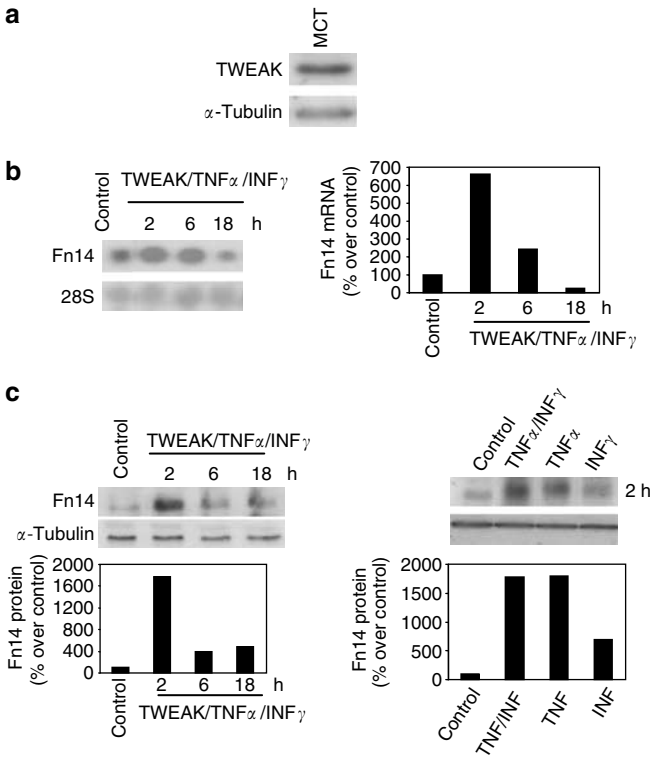

Figure 2 | TWEAK and Fn14 expression in proximal tubular MCT cells. (**a**) Western blot analysis of TWEAK protein expression. (**b**) Northern blot analysis of Fn14 mRNA expression in TWEAK/TNFα/INFγ-treated cells. (**c**) Western blot analysis of Fn14 protein expression in cells treated with cytokines.

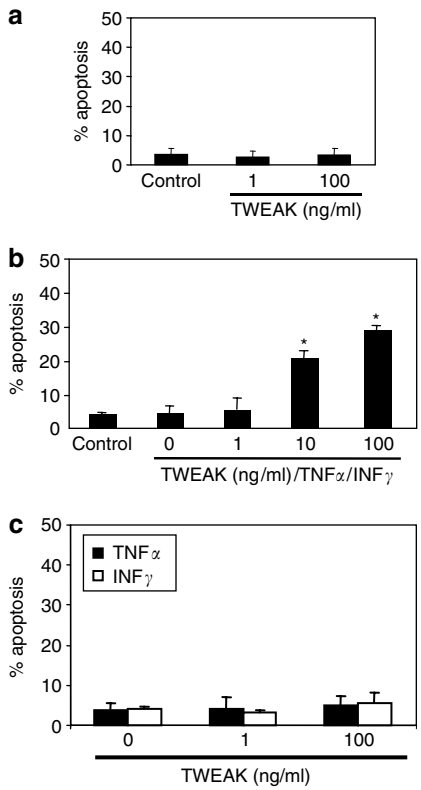

Figure 3 | Modulation of TWEAK-induced apoptosis in MCT cells. Cell death was assessed by flow cytometry of DNA content after culture for 24 h in the presence of recombinant TWEAK and different proinflammatory cytokines. (**a**) TWEAK alone did not induce apoptosis. (**b**) In the presence of TNFα (30 ng/ml) and INFγ (30 U/ml), TWEAK induced apoptosis in a dose-dependent manner. (**c**) Neither INFγ nor TNFα sensitized, by themselves, to TWEAK-induced cell death. Mean (s.d.) of four independent experiments. *$P<0.01$ vs control.

cells (Figure 5). This delayed apoptosis maybe explained by the lethal effect of TNF in MCT cells.[23] That is, TWEAK-induced apoptosis in a proinflammatory milieu is accelerated when compared to that induced by TNFα alone.

MCT cells constitutively express FasL and Fas, and Fas expression is increased by TNFα/INFγ.[12] To rule out an autocrine role for FasL in cell death, cells were cultured with a decoy receptor which antagonizes TWEAK or with FasL blocking antibodies, in the presence of TWEAK/TNFα/INFγ. The decoy receptor protected against apoptosis, whereas blocking FasL was only marginally protective (Figure 6a). However, the decoy receptor did not significantly protect against apoptosis induced by the TNFα/INFγ combination at 48 h (TNFα/INFγ 40±7.8%, TNFα/INFγ/Fn14:Fc 33.5±8.4%, $P=$NS).

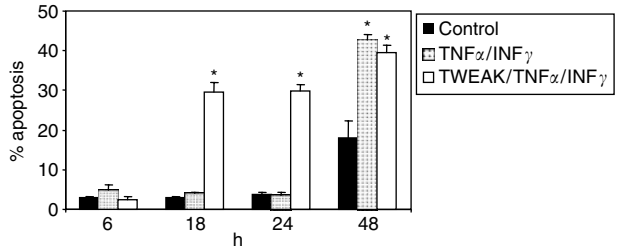

Figure 5 | TWEAK-induced apoptosis in the presence of TNFα/INFγ is time-dependent. Cell death was assessed by flow cytometry of DNA content after culture in the presence of 100 ng/ml TWEAK and TNFα/INFγ. A significant increase in apoptosis was noted at 18 h. In the absence of TWEAK, a significant increase in cell death was delayed for 48 h. Mean (s.d.) of four independent experiments. *$P<0.01$ vs control.

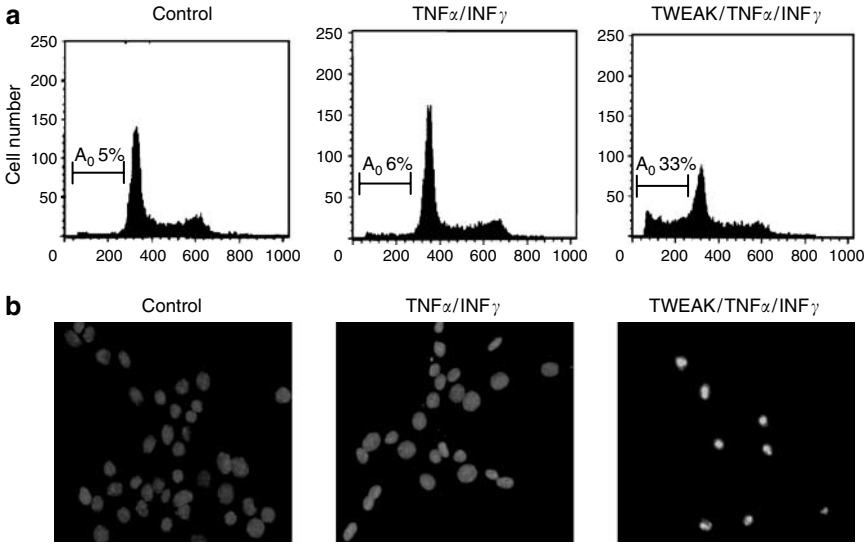

Figure 4 | TWEAK-induced tubular cell death in a proinflammatory environment has features of apoptosis. (**a**) Flow cytometry of DNA content. Note hypodiploid, apoptotic cells (A_0) among those treated with TWEAK/TNFα/INFγ for 24 h. (**b**) Characteristic shrunk, pyknotic, fragmented nuclei are present among 4′,6′-diamidino-2-phenylindole-stained, TWEAK/TNFα/INFγ-treated cells, but not among control or TNFα/INFγ-treated cells (original magnification ×200).

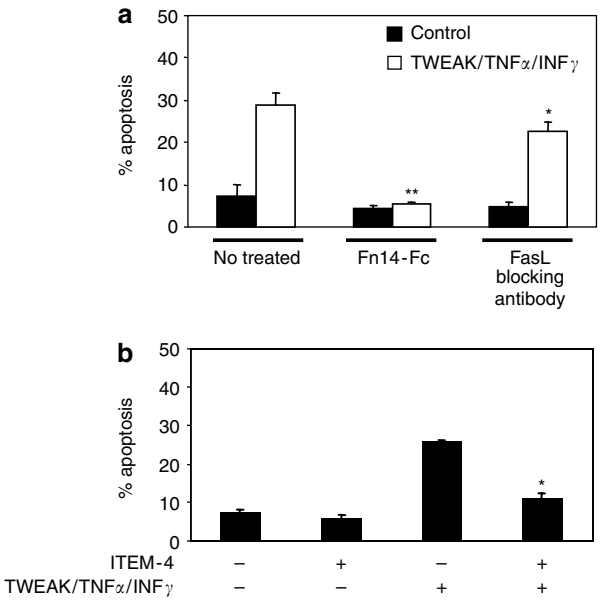

Figure 6 | TWEAK or Fn14 antagonism prevents apoptosis induced by TWEAK//TNFα/INFγ. (**a**) In MCT cells treated with TWEAK/TNFα/INFγ for 24 h, the incubation with decoy receptor for TWEAK prevented apoptosis. A FasL blocking antibody only marginally prevented apoptosis. Apoptosis was assessed by flow cytometry. Mean (s.d.) of three independent experiments. $*P<0.05$ vs Tweak/TNFα/INFγ alone. $**P<0.01$ vs Tweak/TNFα/INFγ alone. (**b**) ITEM-4, a neutralizing anti-Fn14 antibody, prevented apoptosis induced by TWEAK/TNFα/INFγ in MCT cells. Apoptosis was assessed by flow cytometry. Mean (s.d.) of three independent experiments. $*P<0.05$ vs Tweak/TNFα/INFγ alone.

Fn14 mediates TWEAK-induced apoptosis in renal tubular cells

The combination of TWEAK/TNFα/INFγ increased the expression of Fn14 receptor (Figure 2b and c). ITEM-4, an anti-Fn14 neutralizing antibody, prevented TWEAK/TNFα/INFγ-induced apoptosis (Figure 6b). These results suggest that in a proinflammatory environment TWEAK induces apoptosis in the tubular epithelium through Fn14 activation.

TWEAK-induced apoptosis is caspase-dependent

We next investigated caspase involvement in apoptosis induced by TWEAK in the presence of TNFα/INFγ. The combination of cytokines activated caspase-3, -8, and -9 (Figure 7a–c). TWEAK/TNFα/INFγ rapidly induced the appearance of truncated Bid, which preceded caspase-9 and -3 activation (Figure 7c). Bid is a caspase-8 target in death receptor-initiated apoptosis. Caspase-9 processing started at 1 h (Figure 7c). Processing of caspase-3 started at 3 h, as shown by the appearance of caspase-3 cleavage product in Western blots (Figure 7c). Caspase-3 is mainly activated by caspase-8 or caspase-9.[24]

Truncated Bid translocates to the mitochondria and elicits cytochrome *c* release to the cytosol, where the latter activates caspase-9.[25] Cytochrome *c* was released from the mitochondria of cells treated with TWEAK/TNFα/INFγ (Figure 7d and e).

TWEAK does not induce endoplasmic reticulum stress

Growth-arrest and DNA damage (GADD)153 is induced during cell death triggered by endoplasmic reticulum stress.[26] GADD153 expression did not change in tubular cells treated with TWEAK/TNFα/INFγ, unlike in cells treated with the endoplasmic reticulum stress or tunicamycin (Figure 7f). Moreover, cleavage of endoplasmic reticulum-specific caspase-12 was not detected in cells treated with TWEAK/TNFα/INFγ (Figure 7f).

Effect of zVAD on TWEAK-induced cell death and apoptosis

We next studied the effect of caspase inhibition on apoptosis (Figure 8). Benzyloxycarbonyl-Val-Ala-DL-Asp(zVAD)-fluoromethylketone is a broad-spectrum inhibitor of caspases.[27] zVAD-fluoromethylketone inhibited caspase-8 and caspase-3 activity (Figure 7a), caspase-3 processing (Figure 7b), and apoptosis, efficiently blocking DNA hypoploidy, but it did not prevent cell death (Figure 8d). Notably, cells stimulated with TWEAK in the presence of zVAD and cytokines exhibited a necrotic morphology, which was characterized by trypan blue staining (79% of the cells). Similar results were found in cells treated with TWEAK/TNFα/INFγ and caspase-8 inhibitor (data not shown). A role for TWEAK in necrotic death in MCT cells in the presence of caspase inhibition was confirmed, because a TWEAK antagonist prevented the cell death (Figure 8e). In addition, zVAD did not promote cell death in the presence of TNFα/INFγ (Figure 8h).

These results indicate that in a proinflammatory environment TWEAK primarily induces apoptosis in MCT cells, but it can also induce necrosis when caspases are inactivated.

Involvement of reactive oxygen intermediates in TWEAK-induced necrosis

Recently, it has been reported that mitochondrial stress-induced reactive oxygen species were responsible for TNFα or anti-Fas monoclonal antibody-induced necrosis, which was abrogated by antioxidants such as butylated hydroxyanisole (BHA).[28,29] Thus, we tested whether BHA could inhibit TWEAK/TNFα/INFγ-induced necrosis in zVAD-treated cells. The necrotic morphology was completely abrogated by BHA (Figure 8f). These results suggest that caspase inhibition could sensitize MCT cells to TWEAK-induced necrosis via reactive oxygen species-dependent pathway.

DISCUSSION

TWEAK and Fn14 expression has been detected in the normal kidney.[15,21] We now show that both proteins are upregulated in acute tubular injury, suggesting that they may play a role in kidney disease. In the presence of pro-inflammatory cytokines, activation of Fn14 leads to tubular epithelial cell injury. Cell death is associated with caspase-8 activation and recruitment of the mitochondrial pathway of apoptosis. Caspase inhibitors prevent apoptosis but promote oxidative stress and a necrotic form of cell death.

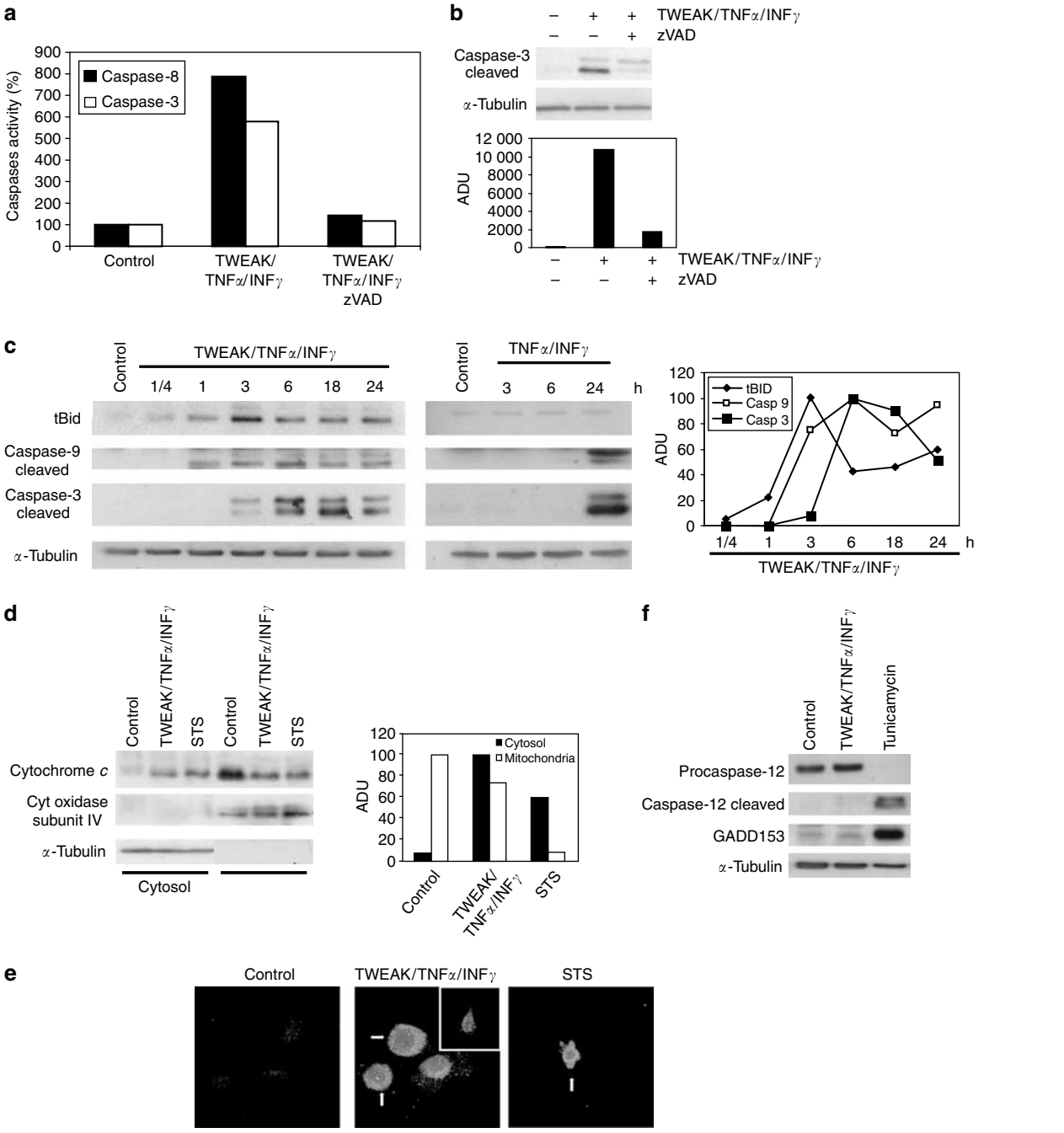

Figure 7 | Activation of caspase-8, -9, and -3, and release of cytochrome *c* from mitochondria in tubular cells. (**a**) Activation of caspases-8 and -3 (activity assay) was inhibited by zVAD (200 μM). (**b**) Incubation with TWEAK/TNFα/INFγ resulted in the appearance of active caspase-3 fragments (Western blot), and zVAD prevented the cleavage of caspase-3. (**c**) Incubation with TWEAK/TNFα/INFγ resulted in the sequential appearance of tBid, and active caspase-9 and -3 fragments (Western blot). Peak caspase-9 activation precedes peak caspase-3 activation. (**d**) Cytochrome *c* is released from mitochondria in MCT cells treated with TWEAK/TNFα/INFγ or 100 nM staurosporine (Western blot). Cytochrome oxidase subunit IV and α-tubulin are controls for fraction separation and loading. (**e**) Cytochrome *c* is released from mitochondria (arrows, confocal microscopy: Cytochrome *c* in green and propidium iodide in red, 24 h) (original magnification $\times$ 320). As a control for cytochrome *c* release, cells were treated with staurosporine (an inducer of the mitochondrial pathway of apoptosis). Inset: early features (30 min) of cytochrome *c* release were observed in some cells. (**f**) Western blot analysis of the expression of GADD153 and caspase-12 cleavage during TWEAK/TNFα/INFγ-, or tunicamycin-induced apoptosis. Tunicamycin is an inducer of endoplasmic reticulum stress. ADU: arbitrary densitometry units.

In different cell types TWEAK may promote cell proliferation or cell death.[15–17,19,20,22,30–34] TWEAK has been shown to promote apoptosis, by itself, in neurons, monocytes, mesangial cells, and certain tumor cell lines.[30–33] By contrast, TWEAK does not induce apoptosis in non-stimulated tubular cells. Nevertheless, the lethal activity of TWEAK may require the coincubation with other agents. TWEAK promotes apoptosis when combined with INFγ in

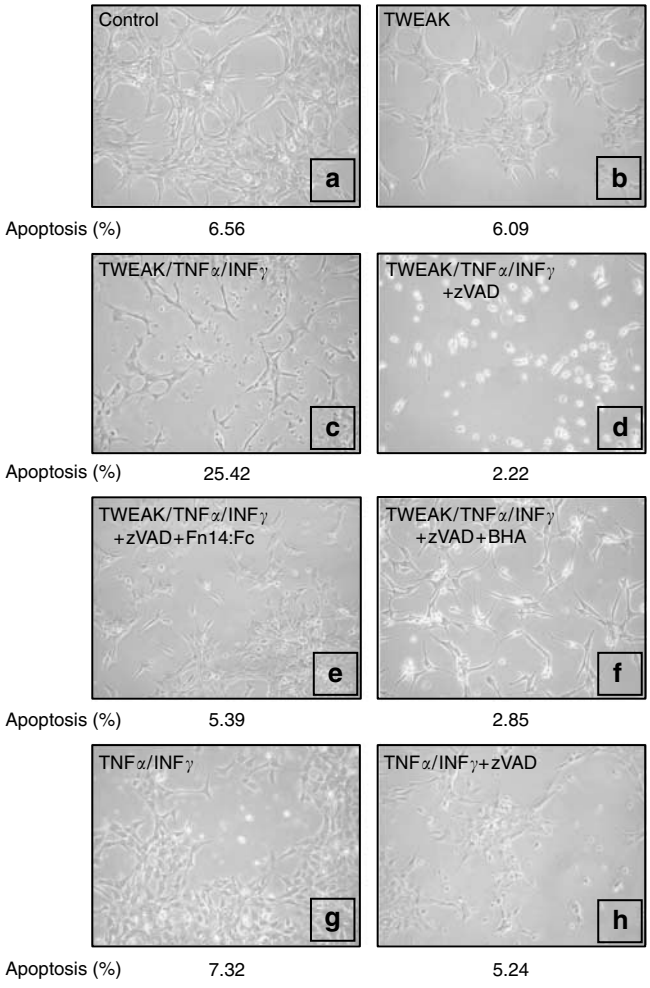

Figure 8 | Effect of zVAD on apoptosis and cell death. (**a**–**h**) MCT cells were exposed for 24 h to different stimuli. Contrast phase microscopic photographs (original magnification × 200). An example representative of at least three independent experiments is shown. Apoptotic cell death at the indicated conditions is also shown. Cells were first photographed and then collected for flow cytometry of DNA content. (**d**) zVAD prevented apoptosis but did not protect from cell death. (**d**) Note cell detachment despite low apoptosis rate in TWEAK/TNFα/INFγ/zVAD treated cells. (**e**, **f**) Both Fn14:Fc and BHA prevented cell death induced by TWEAK/TNFα/INFγ/zVAD. (**h**) zVAD did not promote cell death in the presence of TNFα/INFγ.

some tumor cell lines, whereas others remain resistant even in the presence of this cytokine.[15,22] In tubular epithelial cells, the coincubation with TNFα and INFγ sensitizes to apoptosis induced by TWEAK. This effect was not observed with either cytokine alone. This requirement for both INFγ and TNF is novel. TNFα/INFγ increased Fn14 expression in tubular cells. The early peak of Fn14 expression (2 h) is consistent with previous reports, as Fn14 is an immediate-early response gene.[21] Upregulation of Fn14 expression may underlie the sensitization to apoptosis. In fact, Fn14 transfectants become sensitive to TWEAK-induced death.[22] Alternatively, it has been suggested that certain functions of TWEAK are mediated by a second, not yet characterized receptor.[35] However, a Fn14 blocker prevented the cytotoxicity of the cytokine combination on tubular epithelium. This indicates that Fn14 mediates the lethal effect of TWEAK in tubular cells. An autocrine activation of the FasL/Fas system was also considered as a potential cause of cell death. Our group had previously described that proinflammatory cytokines increase Fas expression in tubular cells.[10] The Fas receptor could theoretically be activated by autocrine FasL, as tubular epithelium constitutively expresses FasL.[10] FasL blocking antibody minimally decreased the rate of cell death induced by TWEAK/TNFα/INFγ, suggesting that the mechanism is indeed active, albeit of little significance in this system.

Next we investigated the intracellular pathways for apoptosis induced by TWEAK in the presence of TNFα/INFγ. We observed activation of caspase-8, proteolysis of Bid, release of cytochrome *c* to the cytosol and activation of caspase-9 and caspase-3. Proteolysis of Bid pre-dated caspase 9, which in turn, pre-dated caspase-3 activation. This suggests that, as it is the case for some cell types in response to activation of death receptors, caspase-8-activated Bid mediated an amplification loop through the mitochondria.[25] Activation of caspases-3, -8, and -9 had previously been observed in some, but not all, tumor cell lines undergoing apoptosis induced by TWEAK.[22,32] However, it is still unclear what adaptors mediate this effect, as Fn14 lacks a death domain.[22] On the other hand, we found no evidence supporting the participation of endoplasmic reticulum stress in the process. The apoptotic endoplasmic reticulum response is preserved in MCT cells exposed to paracetamol or tunicamycin.[36] TNF-α alone may induce a delayed apoptosis in MCT cells, but the time course differs from that of TWEAK, as there is a lag period of 48 h, which was confirmed in cells treated with TNFα/INFγ.[23] In addition, the intracellular molecular mechanisms differ, as cleavage of Bid was not observed in the absence of TWEAK. Most extracellular inputs are not processed in isolation, rather, multiple inputs are perceived by cells in a proinflammatory milieu.[37] It is difficult to assign the final output to a single stimulus.[37] It is conceivable that similar apoptotic pathways engaged by both TNFα and TWEAK in the presence of INFγ cooperate in cell death induction.

Possible therapeutic interventions on tubular cell injury induced by TWEAK were explored. Both TWEAK and Fn14 neutralization were protective. The pancaspase inhibitor zVAD prevented the activation of caspases, and also prevented apoptosis. However, zVAD did not prevent eventual cell death by necrosis. The critical role of TWEAK in necrosis in cells without active caspases was suggested by the fact that zVAD did not promote necrosis in cells exposed only to TNFα/INFγ. In addition, a TWEAK antagonist prevented the necrotic morphology. The induction of necrosis in cells exposed to members of the TNF superfamily when caspases are inhibited had been observed previously.[28,29] The effect of pan caspase inhibition on eventual cell death induced by TWEAK is cell type-specific.[22,32] Caspase inhibition prevented both apoptosis and cell death in a TWEAK-sensitive tumor cell line, whereas this

intervention did not prevent necrosis in a cell line that required INFγ for sensitization to the lethal effect of TWEAK, and it did increase TWEAK-induced cell death in Fn14 transfectants.[32] This latter observation is in accordance with our results suggesting a critical role for Fn14 in the process. The protection afforded by the antioxidant BHA in tubular cells treated with TWEAK/TNFα/INFγ and zVAD suggests the involvement of oxidative stress in the necrotic process that takes place upon caspase inhibition in cytokine-stimulated cells.[38] We can only speculate as to the possible contribution of this caspase-independent pathway for cell death during tubular injury *in vivo*. Even in the absence of therapeutic intervention, there are endogenous caspase inhibitors that may theoretically influence the mode of cell death. However, we do not yet know inhibition of what specific caspase is important for necrotic cell death to proceed. As tubular cell injury, including apoptosis and necrosis, is a feature of the most common form of parenchymal acute renal failure,[4] analyzing the pathways by which as single stimulus may induce either form of cell death will provide new clues to the process that may be used therapeutically.

TNFα is recognized as a key participant in renal injury.[9,13] INFγ is also expressed in the kidney during acute tubular injury.[39] Much less is known about TWEAK/Fn14 expression in renal injury. We now present data showing increased TWEAK expression in an experimental model of acute tubular injury characterized by tubular cell apoptosis and increased TNFα levels.[10] This was associated with a 13-fold increase in Fn14 mRNA and a 2.5-fold increase in tubular cell Fn14 receptor, suggesting a role for TWEAK/Fn14 in the process. Potential sources of TWEAK in the kidney include tubular cells, as shown in the present report, as well as infiltrating leukocytes.[31,40]

In summary, TWEAK induces death of renal tubular epithelial cells under certain microenvironmental conditions, such a proinflammatory milieu composed of TNFα and INFγ. The conditions of increased TWEAK, TNFα, INFγ, and Fn14 expression are observed in experimental acute renal failure. Caspases and mitochondria are involved in the apoptosis of tubular cells induced by TWEAK, but there is no evidence of endoplasmic reticulum stress. The present data identify TWEAK as a cytokine relevant for renal tubular injury. Further studies are needed to fully characterize its role in diverse renal diseases.

MATERIALS AND METHODS

Cells and reagents

MCT cells are a cultured line of proximal tubular epithelial cells that have been extensively characterized.[41] MCT cells were cultured in Rosewell Park Memorial Institute media 1640 (GIBCO, Grand Island, NY, USA), 10% decomplemented fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, in 5% CO_2 at 37°C.[41]

zVAD-fluoromethylketone was from Bachem (Bubendorf, Switzerland), Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoremethylketone (DEVD-fmk) and Z-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone (IETD-fmk) from Calbiochem (San Diego, CA, USA). They were dissolved in dimethylsulfoxide. Final concentration of dimethylsulfoxide in culture did not modulate cell death.[42] Staurosporine (100 nM) and tunicamycin (1 μg/ml) were from Sigma (St Louis, MO, USA).[36,43]

TWEAK and Fn14-Fc (Alexis, Läufelfingen, Switzerland) were dissolved in water. Unless otherwise specified the concentration of TWEAK was 100 ng/ml, Fn14:Fc 5 μg/ml, murine TNFα (Immugenex, Los Angeles, CA, USA) 30 ng/ml and human INF-γ (Immugenex) 30 U/ml. MFL3 neutralizing anti-FasL antibody 10 μg/ml (Pharmingen, San Diego, CA, USA), ITEM-4 neutralizing anti-Fn14 antibody 2.5 μg/ml (Bioscience, San Diego, CA, USA) and BHA 50 μM (Sigma)[12,35,44,45] were also used.

Cell death and apoptosis

Cells (10 000) were seeded in 24-well plates (Costar, Cambridge, MA, USA) in 10% fetal calf serum Rosewell Park Memorial Institute media overnight. Thereafter, they were rested in serum-free medium for 24 h and stimuli were added to subconfluent cells.

Apoptosis was characterized by morphologic and functional criteria. Nuclei of formalin-fixed cells were stained with propidium iodide in the presence of RNAse A to observe the typical morphological changes, as described previously.[12] In addition, nuclei of formalin-fixed cells were stained with 4′,6′-diamidino-2-phenylindole (Vector Laboratories Inc, Burlingame, CA, USA). Both 4′,6′-diamidino-2-phenylindole and propidium iodide staining yielded similar results. For assessment of apoptosis by flow cytometry adherent cells were pooled with spontaneously detached cells, and incubated in 100 μg/ml propidium iodide, 0.05% Nonidet P-40, 10 μg/ml RNAse A in phosphate-buffered saline at 4°C for >1 h. This assay permeabilizes the cells and, thus, it is not based on the known ability of propidium iodide to enter dead cells. The percentage of apoptotic cells with decreased DNA staining (A_0) was counted as described previously.[12]

Western blot

Methods have been described.[12] Primary antibodies were rabbit polyclonal anti-Fn14 1:500,[21] anti-TWEAK (1:500, Santa Cruz Biotechnology, CA, USA), anti-caspase-9 (1:1000, Cell Signalling, Hertfordshire, UK) anti-caspase-12 (1:1000, Cell Signalling), anti-cleaved caspase-3 (1:1000, Cell Signalling), anti-GADD153 (1:500, Santa Cruz), or anti-Bid (1:3000, a gift of SJ Korsmeyer, Dana-Farber Cancer Institute, Boston, MA, USA). Blots were then probed with mouse monoclonal anti-tubulin antibody (1:2000, Sigma) and levels of expression were corrected for minor differences in loading.[12]

Caspase activity

Caspase-3 activity (MBL, Nagoya, Japan) and caspase-8 activity (Sigma) were measured following the manufacturer's instructions.[36,43] In brief, cell extracts (70 μg protein) were incubated with 200 μM DEVD-pNA or Ac-IETD-pNA and pNA light emission was quantified. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase activity.

Cytochrome *c* release from mitochondria

Release of cytochrome *c* from mitochondria to cytosol was measured by Western blot.[36,43] Mitochondria-free cytosolic extracts or mitochondria were electrophoresed on 15% polyacrylamide gels and analyzed by Western blot. Rabbit polyclonal anti-cytochrome *c* (1:500, Santa Cruz Biotechnology) was used. Cytochrome oxidase subunit IV (1:500, Molecular Probes, Leiden, The Netherlands) is

not released from mitochondria during apoptosis, and was used as control. Immunostaining was performed as described previously.[43]

Real-time PCR

Total RNA was extracted by the Trizol method (Life technologies, Carlsbad, CA, USA) and 1 μg was reverse transcribed with the High Capacity complementary DNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR reactions were performed on a ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to manufacturer's protocol and quantitated with the Prism 7000 System SDS Software (Applied Biosystems). Expression levels are given as ratios to glyceraldehyde-3-phosphate dehydrogenase. Pre-developed primer and probe assays were obtained for murine glyceraldehyde-3-phosphate dehydrogenase, Fn14, and TWEAK from Applied Biosystems.

Northern hybridization

Forty micrograms total RNA were processed as described previously.[10] The mouse Fn14 complementary DNA hybridization probe has been described.[21] Membranes were then hybridized to a probe for 28S to adjust for small loading and transfer variations.

Animal models

Balb/c mice (five- to 7-week-old, IFFA-CREDO, Barcelona, Spain) were studied in accord with the NIH Guide for the Care and Use of Laboratory Animals. Folic acid nephropathy is a widely used classical model of acute renal failure characterized by tubular injury, including tubular cell apoptosis, as well as tubular cell proliferation, bone marrow cell dependent regeneration, inflammatory cell infiltration, and mild fibrosis in a chronic phase.[10,44–47] All these features are present in human acute renal failure and represent a good model to study the molecular mechanisms regulating these processes and their therapeutic regulation.[46,47] As it is the case for other forms of acute renal failure, a role of a TNF family member, TNF; has been demonstrated in the disease.[10,23,48] Renal injury was induced by a single intraperitoneal injection of folic acid (Sigma) 250 mg/kg in 0.3 mol/l sodium bicarbonate ($n=5$) or vehicle ($n=5$)[10] and mice were killed 24 h later. Folic acid increased serum creatinine (1.5 ± 0.2 vs 0.3 ± 0.01 mg/dl, $P<0.05$) and was associated with evidence of tubular cell apoptosis, such as the apoptotic morphology (Figure 1e) and internucleosomal DNA degradation.[10]

The kidneys were cold saline perfused *in situ* before removal. One kidney was fixed in buffered formalin, embedded in paraffin and stained with hematoxylin–eosin, and Masson's trichrome or used for immunohistochemistry. The other was snap-frozen in liquid nitrogen for RNA and protein studies.

Immunohistochemistry

Immunohistochemistry was carried out as described previously[10] in paraffin-embedded tissue sections 5 μm thick. Anti-Fn14 antibody was used at a concentration of 10 μg/ml. Anti-TWEAK antibody was used at a concentration of 4 μg/ml (Santa Cruz Biotechnology). For colocalization experiments, specular kidney sections were stained with anti-TWEAK and anti-Fn14 antibody. Sections were counter-stained with Carazzi's hematoxylin. Negative controls included incubation with a nonspecific immunoglobulin of the same isotype as the primary antibody.

Statistics

Results are expressed as mean $\pm$ s.d. Significance at the $P<0.05$ level was assessed by non-parametric Mann–Whitney test for two groups of data and Kruskal–Wallis for three of more groups by means of the SigmaStat statistical software (Jandel, San Rafael, CA, USA).

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REFERENCES

1. Ortiz A. Apoptotic regulatory proteins in renal injury. *kidney Int* 2000; **58**: 467–485.
2. Liano F, Pascual J, the Madrid Acute Renal Failure Study Group. Epidemiology of acute renal failure: a prospective, multicenter, community-based study. *Kidney Int* 1996; **50**: 811–818.
3. Solez K, Morel-Maroger L, Sraer JD. The morphology of 'acute tubular necrosis' in man: analysis of 57 renal biopsies and a comparison with the glycerol model. *Medicine (Baltimore)* 1979; **58**: 362–376.
4. Olsen TS, Olsen HS, Hansen HE. Tubular ultrastructure in acute renal failure in man: epithelial necrosis and regeneration. *Virchows Arch A Pathol Anat Histopathol* 1985; **406**: 75–89.
5. Ortiz A, Justo P, Sanz A *et al.* Targeting apoptosis in acute tubular necrosis. *Biochem Pharmacol* 2003; **66**: 1589–1594.
6. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001; **104**: 487–501.
7. Bodmer JL, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. *Trends Biochem Sci* 2002; **27**: 19–26.
8. Ortiz A, Lorz C, Egido J. New kids in the block: the role of FasL and Fas in kidney damage. *J Nephrol* 1999; **12**: 150–158.
9. Ortiz A, Gonzalez-Cuadrado S, Bustos C *et al.* Tumor necrosis factor and glomerular damage. *J Nephrol* 1995; **8**: 27–34.
10. Ortiz A, Lorz C, Catalán MP *et al.* Expression of apoptosis regulatory proteins in tubular epithelium stressed in culture or following acute renal failure. *Kidney Int* 2000; **57**: 969–981.
11. Ortiz-Arduan A, Danoff TM, Kalluri R *et al.* Regulation of Fas and Fas ligand expression in cultured murine renal cells and in the kidney during endotoxemia. *Am J Physiol* 1996; **241**: F1193–F1201.
12. Lorz C, Ortiz A, Justo P *et al.* Proapoptotic Fas ligand is expressed by normal kidney tubular epithelium and injured glomeruli. *J Am Soc Nephrol* 2000; **11**: 1266–1277.
13. Misseri R, Meldrum DR, Dinarello CA *et al.* TNF-alpha mediates obstruction-induced renal tubular cell apoptosis and proapoptotic signaling. *Am J Physiol Renal Physiol* 2005; **288**: F406–F411.
14. Hamar P, Song E, Kokeny G *et al.* Small interfering RNA targeting Fas protects mice against renal ischemia-reperfusion injury. *Proc Natl Acad Sci USA* 2004; **101**: 14883–14888.
15. Chicheportiche Y, Bourdon PR, Xu H *et al.* TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem* 1997; **272**: 32401–32410.
16. Wiley SR, Winkles JA. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. *Cytokine Growth Factor Rev* 2003; **14**: 241–249.
17. Campbell S, Michaelson J, Burkly L *et al.* The role of TWEAK/Fn14 IN the pathogenesis of inflammation and systemic autoimmunity. *Front Biosci* 2004; **9**: 2273–2284.
18. Chicheportiche Y, Chicheportiche R, Sizing I *et al.* Proinflammatory activity of TWEAK on human dermal fibroblasts and synoviocytes: blocking and enhancing effects of anti-TWEAK monoclonal antibodies. *Arthritis Res* 2002; **4**: 126–133.
19. Lynch CN, Wang YC, Lund JK *et al.* TWEAK induces angiogenesis and proliferation of endothelial cells. *J Biol Chem* 1999; **274**: 8455–8459.
20. Wiley SR, Cassiano L, Lofton T *et al.* A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. *Immunity* 2001; **15**: 837–846.

21. Meighan-Mantha RL, Hsu DK, Guo Y *et al.* The mitogen-inducible Fn14 gene encodes a type I transmembrane protein that modulates fibroblast adhesion and migration. *J Biol Chem* 1999; **274**: 33166–33176.
22. Nakayama M, Ishidoh K, Kojima Y *et al.* Fibroblast growth factor-inducible 14 mediates multiple pathways of TWEAK-induced cell death. *J Immunol* 2003; **170**: 341–348.
23. Justo P, Sanz A, Lorz C *et al.* Expression of Smac/Diablo in tubular epithelial cells and during acute renal failure. *Kidney Int* 2003; **64**(Suppl 86): 52–56.
24. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998; **281**: 1312–1316.
25. Li H, Zhu H, Xu CJ *et al.* Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998; **94**: 491–501.
26. Zinszner H, Kuroda M, Wang XZ *et al.* CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Gene Dev* 1998; **12**: 982–995.
27. Garcia-Calvo M, Peterson EP, Leiting B *et al.* Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem* 1998; **273**: 32608–32613.
28. Vercammen D, Brouckaert G, Denecker G *et al.* Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J Exp Med* 1998; **188**: 919–930.
29. Khwaja A, Tatton L. Resistance to the cytotoxic effects of tumor necrosis factor alpha can be overcome by inhibition of a FADD/caspase-dependent signaling pathway. *J Biol Chem* 1999; **274**: 36817–36823.
30. Potrovita I, Zhang W, Burkly L *et al.* Tumor necrosis factor-like weak inducer of apoptosis-induced neurodegeneration. *J Neurosci* 2004; **24**: 8237–8244.
31. Kaplan MJ, Lewis EE, Shelden EA *et al.* The apoptotic ligands TRAIL, TWEAK, and Fas ligand mediate monocyte death induced by autologous lupus T cells. *J Immunol* 2002; **169**: 6020–6029.
32. Nakayama M, Ishidoh K, Kayagaki N *et al.* Multiple pathways of TWEAK-induced cell death. *J Immunol* 2002; **168**: 734–743.
33. Campbell S, Burkly LC, Gao HX *et al.* Proinflammatory effects of TWEAK/Fn14 interactions in glomerular mesangial cells. *J Immunol* 2006; **176**: 1889–1898.
34. Brown SA, Richards CM, Hanscom HN *et al.* The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor-kappaB activation. *Biochem J* 2003; **371**: 395–403.
35. Polek TC, Talpaz M, Darnay BG *et al.* TWEAK mediates signal transduction and differentiation of RAW264.7 cells in the absence of Fn14/TWEAKR. Evidence for a second TWEAK receptor. *J Biol Chem* 2003; **278**: 32317–32323.
36. Lorz C, Justo P, Sanz A *et al.* Paracetamol-induced renal tubular injury: a role for endoplasmic reticulum stress. *J Am Soc Nephrol* 2004; **15**: 380–389.
37. Janes KA, Albeck JG, Gaudet S *et al.* A systems model of signaling identifies a molecular basis set for cytokine-induced apoptosis. *Science* 2005; **310**: 1646–1653.
38. Cauwels A, Janssen B, Waeytens A *et al.* Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. *Nat Immunol* 2003; **4**: 387–393.
39. Goes N, Urmson J, Ramassar V *et al.* Ischemic acute tubular necrosis induces an extensive local cytokine response. Evidence for induction of interferon-gamma, transforming growth factor-beta 1, granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-10. *Transplantation* 1995; **59**: 565–572.
40. Nakayama M, Kayagaki N, Yamaguchi N *et al.* Involvement of TWEAK in interferon gamma-stimulated monocyte cytotoxicity. *J Exp Med* 2000; **192**: 1373–1380.
41. Haverty TP, Kelly CJ, Hines WH *et al.* Characterization of a renal tubular epithelial cell line which secretes the autologous target of autoimmune experimental interstitial nephritis. *J Cell Biol* 1988; **107**: 1359–1368.
42. Ortiz A, Lorz C, Catalán MP *et al.* Cyclosporine A induces apoptosis in murine tubular epithelial cells: role of caspases. *Kidney Int Suppl* 1998; **68**: S25–S29.
43. Justo P, Lorz C, Sanz A *et al.* Intracellular mechanisms of cyclosporin A-induced tubular cell apoptosis. *J Am Soc Nephrol* 2003; **14**: 3072–3080.
44. Ortega A, Ramila D, Ardura JA *et al.* Role of parathyroid hormone-related protein in tubulointerstitial apoptosis and fibrosis after folic Acid-induced nephrotoxicity. *J Am Soc Nephrol* 2006; **7**: 1594–1603.
45. Fang TC, Alison MR, Cook HT *et al.* Proliferation of bone marrow-derived cells contributes to regeneration after folic acid-induced acute tubular injury. *J Am Soc Nephrol* 2005; **6**: 1723–1732.
46. Doi K, Okamoto K, Negishi K *et al.* Attenuation of folic acid-induced renal inflammatory injury in platelet-activating factor receptor-deficient mice. *Am J Pathol* 2006; **68**: 1413–1424.
47. Dai C, Yang J, Liu Y. Single injection of naked plasmid encoding hepatocyte growth factor prevents cell death and ameliorates acute renal failure in mice. *J Am Soc Nephrol* 2002; **3**: 411–422.
48. Wan B, Hao L, Qiu Y *et al.* Blocking tumor necrosis factor-alpha inhibits folic acid-induced acute renal failure. *Exp Mol Pathol* 2006; 2 April (E-pub ahead of print).