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TWEAKing renal injury

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1. ABSTRACT

TWEAK is a recently identified cytokine of the TNF superfamiliy. Through activation of the Fn14 receptor, TWEAK regulates cell proliferation, cell death and inflammation. Recent studies show increased TWEAK and Fn14 expression in tubular cells during acute kidney injury as well as elevated urinary TWEAK levels in patients with active lupus nephritis. Furthermore, glomerular mesangial cells and renal tubular epithelial cells express the Fn14 receptor under the regulation of proinflammatory cytokines. TWEAK weakly increases cell death and promotes secretion of inflammatory mediators in non-stimulated mesangial cells. In addition, in a proinflammatory milieu, TWEAK induces apoptosis of mesangial and tubular cells. The available data suggest that TWEAK is a new player in kidney injury both at the glomerular and tubulointerstitial levels and might be a target for therapeutic intervention.

2. INTRODUCTION

The verb "to tweak" has several meanings. The Oxford English dictionary identifies two main definitions: twist or pull with a small but sharp movement and improve by making fine adjustments. Recent advances in the understanding of tumor necrosis factor-like weak inducer of apoptosis (TWEAK, Apo3L, TNFSF12) actions in the kidney suggest that the second meaning more closely represents the role of TWEAK in kidney injury. As detailed below, TWEAK has several actions that may modulate renal injury, including modulation of cell death, cell proliferation and inflammation. Interestingly the precise effect of TWEAK over kidney cells is modulated by the cell microenvironment, allowing it to fine-tune the renal response to injury.

3. TWEAK AND Fn14

3.1. Structure

TWEAK was identified in 1997 as a member of the tumor necrosis factor superfamily (TNFSF) of structurally-related cytokines (1). The human TWEAK gene is located at chromosomal position 17p13.1 and encodes a 249-amino acid (aa) type II transmembrane glycoprotein (30kDa) containing a C-terminal extracellular domain (206-aa), a transmembrane domain (25-aa), and an N-terminal intracellular domain (18-aa). The intracellular domain contains a putative serine phosphorylation site. The extracellular domain presents the receptor-binding site where a glycosylation consensus sequence has been described at position 139. Most cells express two TWEAK isoforms, a membrane-bound form (mTWEAK) and a cleaved soluble form (sTWEAK) of 156-aa (18kDa) that results from proteolysis of full-length TWEAK. TWEAK contains an extracellular TNF homology domain via which it associates into non-covalently linked homotrimers that mediate its biological effects (2, 3). Studies assessing TWEAK functions were conducted using recombinant sTWEAK. It is presently unknown whether mTWEAK is biologically active. However, it has recently been reported that mTWEAK, but not sTWEAK, may enter the cell nucleus (4). The functional significance of this observation is currently unknown (4).

TWEAK receptor was initially reported to be the TNFSF member named DR3 (5). However, different subsequent studies failed to confirm a TWEAK-DR3 interaction. Moreover, other reports have demonstrated that TWEAK can bind to different cell types that do not express DR3 (6-7), indicating that other protein/s were the receptor/s of TWEAK. Thus, Wiley *et al.* identified in 2001 a novel receptor for TWEAK that was named TWEAK receptor (TWEAKR) (8). The DNA sequence analysis demonstrated that TWEAKR was identical to human fibroblast growth factor-inducible 14 (Fn14, TNFRSF12A), described by Winkles´s group several years earlier (9), and it was confirmed that TWEAK binds to Fn14 (10). The human Fn14 gene is located at the chromosomal position 16p13.3 (11) and encodes a 129-aa type I transmembrane protein (14kDa). Fn14 is synthesized with an N-terminal signal peptide which is processed into a 102-aa mature form of the protein, making it the smallest TNF receptor (TNFR) superfamily member identified to date (2). The Fn14 extracellular domain (53-aa) presents the TWEAK-binding site in a cysteine-rich region (12). The intracellular domain (29-aa) of Fn14 contains a TNFR-associated factor (TRAF)-binding site with three threonines that could be potentially phosphorylated and induce TRAF binding and subsequent transmission of TWEAK signaling (13). Trimeric TWEAK binds to Fn14 monomers, promoting receptor trimerization and further signal transduction (13-16).

Human and murine TWEAK are closely related, with a 93% homology in the receptor-binding domain. Furthermore, human and murine Fn14 have a 90% homology in their overall sequences. This detail is of importance since it has recently been published that TWEAK does not cross-react with any other members of the TNF or TNFR superfamilies, its interaction being specific for Fn14. In addition, human TWEAK can bind to murine Fn14 and vice-versa (17). Although Fn14 is the only characterized TWEAK receptor, Polek *et al.* have recently proposed that some biological effects of TWEAK may be mediated independently of its interaction with Fn14 in RAW cells (monocytes/macrophages) (18). This suggests the presence of additional receptors or receptor-independent TWEAK signaling.

3.2. Expression

TWEAK is widely expressed, and can be found at higher levels in pancreas, intestine, heart, brain, lung, ovary and skeletal muscle, and at lower levels in liver and kidney (1, 19). Furthermore, TWEAK mRNA has been detected in different cultured cells of potential interest in renal injury such as human peripheral blood mononuclear cells (PBMC), mouse macrophages, human endothelial cells (ECs) and smooth muscle cells (SMCs). On the other hand, Fn14 mRNA has been detected in kidney, heart, lung, skin, spleen, brain, placenta, ECs, SMCs, PBMC, fibroblasts, monocyte/macrophages, and astrocytes (9-11). However, discrepancies have been noted between TWEAK or Fn14 mRNA expression and protein expression. In this sense, although TWEAK mRNA is detected in lymphocytes, the cytokine is not found on the membrane of these cells. Furthermore, although SMCs express Fn14 at the mRNA level (8), the protein was not detected in quiescent SMCs in culture (20). A low sensitivity of the protein detection method could contribute to these observations.

The mechanisms of regulation of TWEAK expression are poorly understood. There are examples of TWEAK expression regulation *in vivo*. Thus, the levels of TWEAK mRNA were initially reported to be reduced in mice undergoing both acute (induced by lipopolysaccharide injection) or chronic (autoimmune pathologies like erythematosus lupus or hemolytic anemia) inflammatory processes in numerous tissues and peritoneal macrophages (21). However, elevated levels were found in other inflammatory conditions such as experimental autoimmune encephalitis (22). *In vitro*, it has been verified that the treatment with INF-gamma induces TWEAK expression (23).

Fn14 initially was identified as a gene whose transcription was quickly induced by FGF-1 (fibroblast growth factor-1) in murine fibroblasts (9). In addition, Fn14 is also induced by FGF-2, serum or by the treatment with forbol esters (9) in murine fibroblasts, human lung fibroblasts (11), and rat aorta SMCs (8). Fn14 is expressed at high levels in migrating glioma cells *in vitro* and invading glioma cells *in vivo* (14). *In vivo*, the induction of Fn14 has been studied in models of repair of damaged tissue. An increase in mouse liver Fn14 mRNA levels was observed during the regeneration process induced by partial hepatectomy (11). Another study demonstrated that Fn14 expression is increased *in vivo* following arterial injury (8). The last one shows an upregulation of Fn14 levels in dorsal root ganglia after sciatic nerve transaction, suggesting that Fn14 contributes to nerve regeneration (24).

As far as renal disease is concerned, TWEAK and Fn14 expression is upregulated in experimental models of acute tubular injury and renal inflammation (25). The potential sources of TWEAK in the kidney include infiltrating monocytes and T lymphocytes (26, 27). In particular T cells from lupus patients express TWEAK (26). Other renal sources of TWEAK are possible, since the cytokine is expressed in the normal kidney. Indeed, cultured tubular epithelial cells express TWEAK and Fn14 (25). In addition, it has been reported recently that mesangial cells also express TWEAK and Fn14 (28).

3.3. Signaling pathway

The signaling induced by TNF superfamily receptors usually involves the presence of death domains in their cytoplasmatic tail. However, Fn14 cytoplasmatic tail is too short to have a death domain but instead displays a single TRAF-binding site that associates with TRAFs (TRAF1, 2, 3 and 5) (3, 13, 29). In several reports it has been shown that mutated receptors missing TRAF-binding site are unable to signal (13-16). Association of TRAFs is known to activate nuclear factor (NF)kappaB associated intracellular signaling pathways. This is in agreement with the observations of previous studies, where TWEAK activated the NF-kappaB signaling cascade in several cell lines, including kidney mesangial cells (13,14,16,28-35). Furthermore, the induction of NF-kappaB-regulated genes such as IL-6, IL-8, RANTES and ICAM-1 in TWEAK-treated cell lines also points to a role for the NF-kappaB pathway in transducing TWEAK signaling intracellularly (10), as indeed confirmed by several investigators (3). However, the blocking of chemokine secretion in TWEAK-treated astrocytes by an inhibitor of p38 (36) indicates that additional signaling pathways may also be involved. Thus, Donohue *et al.* (30) detected TWEAK-stimulated extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) phosphorylation in human ECs.

4. TWEAK BIOLOGICAL ACTIVITY

Recent studies have revealed that the TWEAK-Fn14 ligand-receptor pair likely plays an important role in a variety of cellular processes and in the pathogenesis of several human diseases, including atherosclerosis, stroke, rheumatoid arthritis and cancer (37). TWEAK has been reported to stimulate cell proliferation (30, 38-41), survival (42), migration (32, 39, 43), cell growth (2) and apoptosis (1, 7, 35, 44-46). TWEAK can also promote (18) or inhibit (23, 34, 47) cellular differentiation. Finally, TWEAK treatment of numerous cell types has been shown to induce the expression of various pro-inflammatory molecules (1, 21, 28, 31-33, 36, 39, 48). TWEAK also has activity when administered *in vivo*. TWEAK can stimulate blood vessel formation (angiogenesis) (38, 49) and regulates neurovascular unit permeability (50). More recently, it was reported that injection of TWEAK to mice significantly up-regulates the kidney gene expression of monocyte chemotactic protein (MCP)-1, and INFgamma-induced protein (IP)-10. This effect is mediated by the Fn14 receptor, demonstrating for the first time that TWEAK has pro-inflammatory activity *in vivo* (28).

5. TWEAK AND CELL DEATH

Several members of the TNF ligand superfamily directly induce apoptotic cell death, including TRAIL, Fas, TNF-alpha and lymphotoxin. TWEAK shares this property. Indeed, the name derives from its weak capacity to induce apoptosis. TWEAK-induced apoptosis of tumor cell lines requires long incubation time, high ligand concentration, and co-incubation with sensitizing agents such as INFgamma. The initial observation of TWEAK-induced apoptosis was made in INFgamma-treated human HT29 cells (adenocarcinoma cell line)(1). TWEAK also induced weakly apoptosis in INFgamma-treated gastric adenocarcinoma KATO-III cells (7) and cycloheximide pretreated MCF-7 breast carcinoma cells (19). By contrast, Kym-1 rhabdomyosarcoma cells and HSC3 oral squamous carcinoma cells are sensitive to TWEAK in the absence of cofactors (7). In addition, TWEAK mediates INFgamma-stimulated human monocyte cytotoxicity against various tumor cell lines (27) and the anti-tumor effect of tumor-infiltrating macrophage (51).

TWEAK may also induce cell death in non-tumor cells. TWEAK has been implicated in apoptosis of monocytes and macrophages induced by activated $CD4^+$ positive T cells (52). Moreover, TWEAK alone may induce apoptosis in primary neurons (35). In the kidney, TWEAK weakly induces apoptosis in non-stimulated mesangial cells and this effect is magnified in the presence of INFgamma (28). By contrast, non-stimulated tubular epithelial cells are resistant to TWEAK-induced apoptosis and the lethal effect only becomes apparent in the presence of several (not one) inflammatory mediators, such as INFgamma and TNF-alpha combination (25).

The signaling pathways leading to cell death remains poorly understood and there is evidence that it may differ with cell type and the cell microenvironment. The lack of a DD suggests that direct recruitment of DD-containing adaptor proteins is not the primary mode of inducing cell death. TRAFs and TRAF-binding proteins such as receptor-interacting protein (RIP) and TNFR-associated DD (TRADD) may be involved (53). TWEAK can directly induce apoptosis, via TWEAK/Fn14 interaction, in tumor HSC3 cells and INFgamma treated HT29 cells and also in primary culture neurons (46,7,35). On the other hand, TWEAK may induce apoptosis indirectly, through recruitment of endogenous TNF-alpha and TNF receptor 1: TNF-alpha is required in TWEAK-induced cell death of Kym-1 rhabdomyosarcoma cells (44).

Evidence from the use of caspase inhibitors also points to several pathways for cell death. The pan-caspase inhibitor z-VAD-fmk abrogates both the TWEAK-induced apoptosis and cell death in HSC3 cells (7). However, in HT29 cells and renal tubular cells, z-VAD-fmk prevents TWEAK-induced apoptosis but sensitizes to death by necrosis (7 and 25). The induction of necrosis in cells exposed to members of the TNF superfamily when caspases are inhibited had been previously observed and oxidative stress is involved in this necrotic process (54, 55).

Antioxidants such as BHA prevents necrotic cell death induces by TWEAK when caspases are inhibited (46, 25).

6. THE ROLE OF TWEAK AND FN14 IN RENAL INJURY

The biological actions of TWEAK have been explored in glomerular mesangial cells and renal epithelial cells (25,28). In addition, increased expression of both TWEAK and Fn14 has been observed during acute kidney injury (25). TWEAK induces apoptosis in mesangial and tubular cells and may also promote renal inflammation (25, 28).

The TWEAK/Fn14 interaction can activate NFkappaB pathway in diverse cell types (13,29,34,50). Activation of NFkappaB by TNF superfamily receptors frequently leads to protection from cell death. This was also the case for TWEAK in glioma cells, where TWEAK promoted survival through up-regulation of BCL-XL and BCL-W mRNA and protein levels (14). However, in at least one case, NFkappaB mediated TWEAK induced-cell death: inhibition of NFkappaB reduced neuronal cell death promoted by TWEAK (35)

6.1. Glomerular injury

The fact that mesangial cells express Fn14 and respond to stimulation by TWEAK suggests that this cytokine might have a role in glomerular injury. TWEAK weakly induced apoptosis in these cells (28). The effect is more pronounced in the presence of INFgamma, suggesting that it may be exacerbated in the presence of glomerular inflammation. This action is reminiscent of the ability of FasL to promote mesangial cell apoptosis (56,57). FasL induces apoptosis in unprimed cultured mesangial cells and cell death in increased in primed cells. In addition, injection of Fas agonists triggered mesangial cell apoptosis and glomerular injury characterized by proteinuria and hematuria *in vivo* (57). However, the *in vivo* consequences of TWEAK-induced mesangial cell apoptosis have not been explored and expression of TWEAK and Fn14 in glomerular injury has not been characterized.

In addition, TWEAK induced the expression of inflammatory mediators such CCL2/MCP-1, CCL5/RANTES, CXCL10/IP10 and CXCL1/KC in cultured mesangial cells (28). The induced levels of chemokines were comparable to those found following mesangial cell exposure to classical proinflammatory stimuli such as TNF-alpha and IL-1beta, but IL-1beta, IL-6, or TNF-alpha do not mediate the proinflammatory effects of TWEAK. CXCL11, CXCR5, and VCAM-1 were up-regulated by TWEAK as well. TWEAK significantly up-regulates kidney CCL2/MCP-1 and CXCL10/IP-10 mRNA *in vivo*. However, the nature of the cells responsible for these *in vivo* effects was not addressed. Chemokines such as CCL2/MCP-1 and CCL5/RANTES play prominent roles in inflammatory glomerular diseases by recruiting monocytes and activated T cells (58). It remains to be addressed whether increased mRNA expression was associated with a concomitant increase in chemokine levels, whether glomerular inflammation was a consequence of changes in transcription of chemokines and whether blocking of TWEAK prevents glomerular inflammation.

6.2. Acute kidney injury

Tubular cells compose most of the mass of the functioning kidneys. Loss of renal tubular cells characterizes both acute and chronic failure of renal function (59). In the course of injury tubular cells may be shed, die or differentiate into fibroblasts, and all of these mechanisms may contribute to tubular cell loss. In human studies tubular cell death was the best histopathological correlate of renal dysfunction during acute kidney injury (60,61). The key role of tubular cell death in the genesis of acute (62-65).

Although there is no information on TWEAK/Fn14 and human acute kidney injury, recent data have emerged from an animal model that closely mimics some features of the human condition. An overdose of folic acid induces acute renal failure in humans (66). In mice it causes an acute renal failure characterized by tubular cell apoptosis, compensatory tubular cell proliferation leading to regeneration, inflammatory cell infiltration and mild fibrosis in a chronic phase (67-68). In this model, expression of both TWEAK and Fn14 increased both at the mRNA and protein level (25). There was a 13-fold increase in Fn14 mRNA and a 2.5-fold increase in tubular cell Fn14 receptor. Fn14 was localized to injured, dilated tubules (Figure 1). Both proximal and distal tubules expressed Fn14 (25).

As a part of a generalized effort to unravel the role of TWEAK/Fn14 in acute kidney injury, our group has first addressed the modulation of tubular cell death. In non-primed cultured tubular cells TWEAK did not induce apoptosis (Figure 2). However, during acute kidney injury multiple cytokines are released, including TNF-alpha and INFgamma (67,69). It seems reasonable to explore the result of the concomitant presence of several of them (70). The coincubation with either of these cytokines and TWEAK did not promote cell death. However, the concomitant presence in the cell culture media of the three of them resulted in apoptotic cell death. This requirement for both INFgamma and TNFalpha in order to sensitize to cell death in the presence of TWEAK is novel. TNF-alpha /INFgamma increased Fn14 expression in tubular cells. The early peak of Fn14 expression (2h) is consistent with previous reports, since Fn14 is an immediate-early response gene (9). Upregulation of Fn14 expression may underlie the sensitization to apoptosis. In fact, Fn14 transfectants become sensitive to TWEAK induced death (46). A Fn14 blocker prevented the cytotoxicity of the cytokine combination on tubular epithelium, indicating that Fn14 mediates the lethal effect of TWEAK in tubular cells. However, the level of Fn14 expression is not the only mechanism involved, since INFgamma or TNF-alpha alone also increase Fn14 expression but do not sensitize to cell death. 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 (71). The

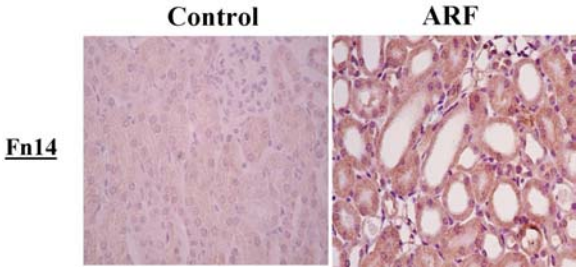

Figure 1. Increased immunoreactive Fn14 in tubular cells during acute kidney injury (ARF). Tubular cell injury was induced in mice by a single folic acid overdose. Note dilated tubuli in the injured kidney.

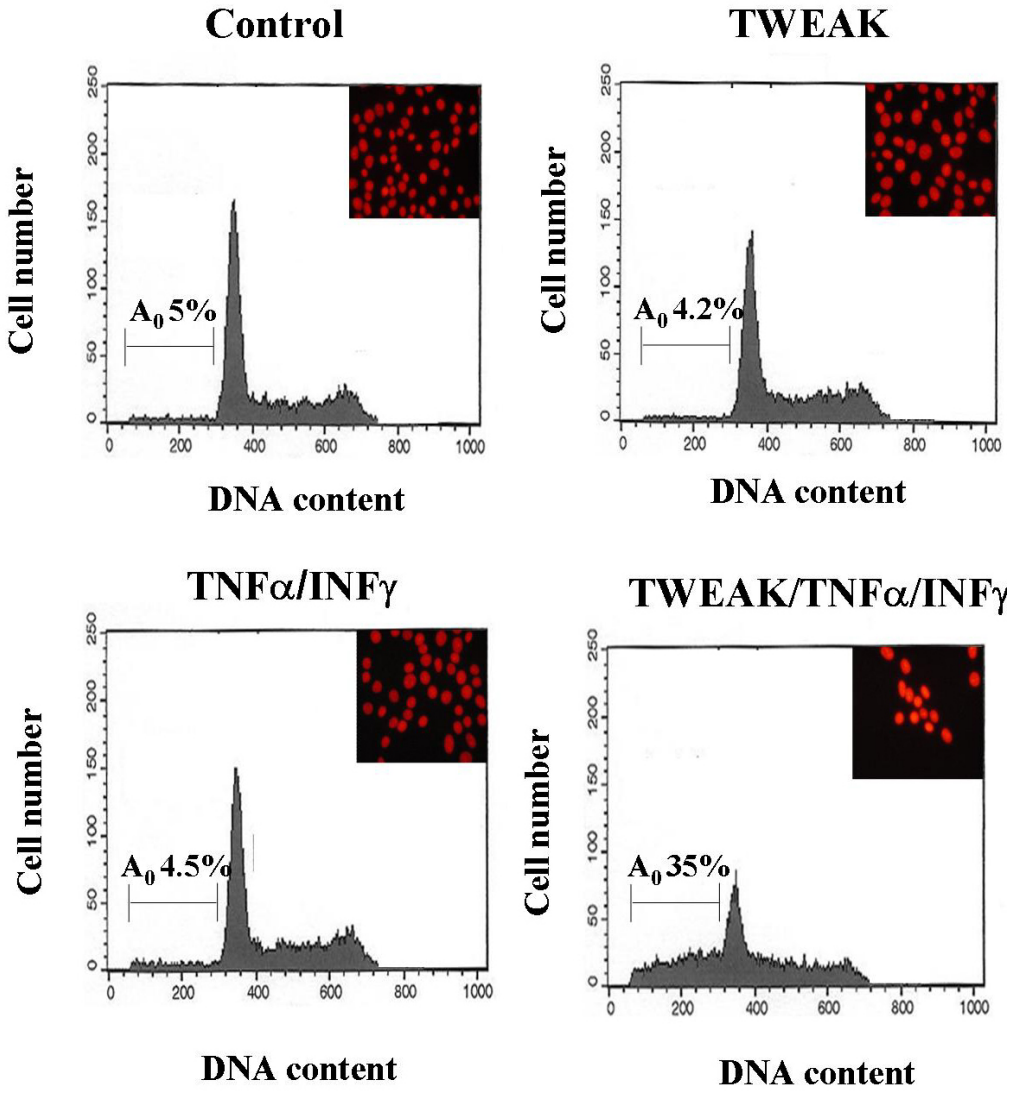

Figure 2. When combined, TNF-alpha (30 ng/ml), INFgamma (30 U/ml), and TWEAK (100 ng/ml) induce apoptosis in cultured renal tubular epithelial cells. Apoptosis is prevented by caspase inhibition. Flow cytometry diagrams of cell DNA content. Ao hypodiploid cells are apoptotic. Inset: propidium iodide nuclear staining shows pyknotic, shrunk, bright nuclei characteristic of apoptosis among cells treated with TWEAK/ TNF-alpha / INFgamma for 24h.

Fas receptor could theoretically be activated by autocrine FasL, as tubular epithelium constitutively expresses FasL. FasL blocking antibody minimally decreased the rate of cell death induced by TWEAK/TNF/INFgamma, suggesting that the mechanism is indeed active, albeit of little significance in this system.

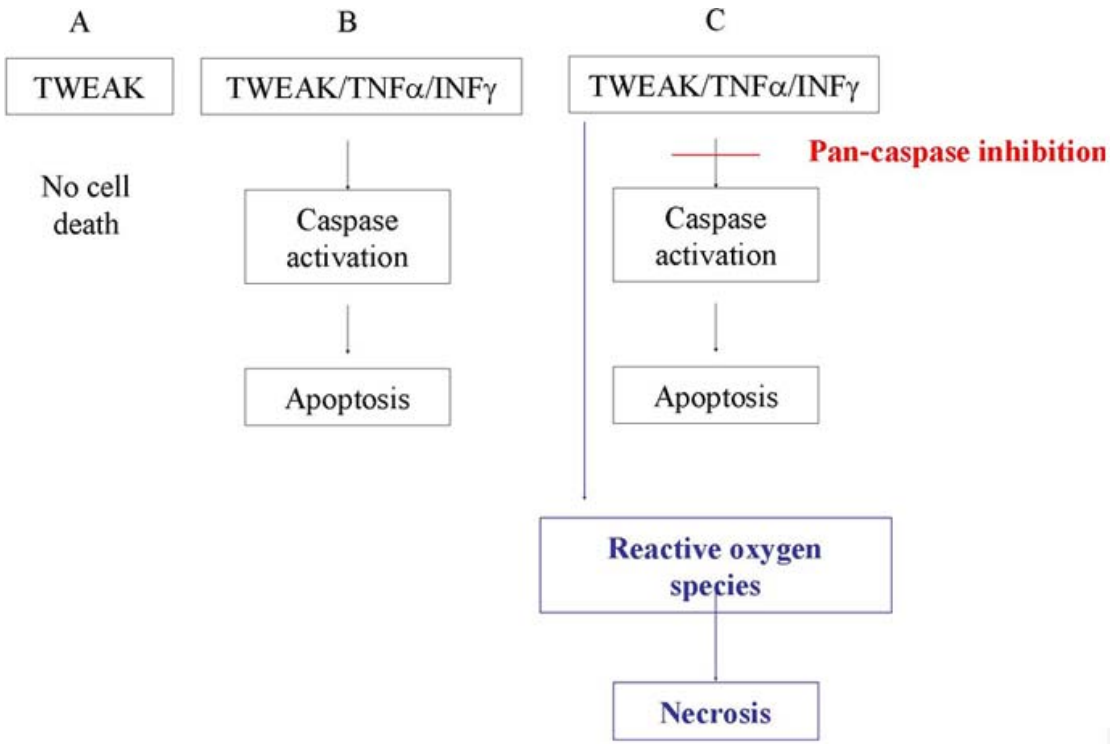

Figure 3. TWEAK/ TNF-alpha /INFgamma activate caspases and promote apoptosis in cultured renal tubular epithelial cells. Inhibition of caspases by the broad-spectrum inhibitor zVAD prevents apoptosis but leads to oxidant-mediated necrosis.

TWEAK in the presence of TNF-alpha /INFgamma resulted in 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. The possible role of caspase-2, which may also cleave Bid, was not explored. Activation of caspases-3, -8 and -9 had previously been observed in some, but not all, tumor cell lines undergoing apoptosis induced by TWEAK. 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 (62).

Most extracellular inputs are not processed in isolation, rather, multiple inputs are perceived by cells in a proinflammatory milieu (70). Thus, it is difficult to assign the final output to a single stimulus. It is conceivable that similar apoptotic pathways engaged by both TNF-alpha and TWEAK in the presence of INFgamma cooperate in cell death induction. Indeed, TNF-alpha may induce a delayed apoptosis in MCT cells, but the time-course differs from that of TWEAK, as there is a lag period of 48h, which was confirmed in cells treated with TNF-alpha /INFgamma (67). In addition, the intracellular molecular mechanisms differ between death induced by TNF alone and by the cytokine combination. Cleavage of Bid was not observed in the absence of TWEAK (25). The pancaspase inhibitor zVAD prevented the activation of caspases, and also prevented apoptosis induced by TWEAK/TNF-alpha /INFgamma (25). However, zVAD transformed the mode of cell death to necrosis and even increased the rate of cell death. Although the induction of necrosis in cells exposed to members of the TNF superfamily, including TNF-alpha, when caspases are inhibited had been previously observed (55), zVAD did not promote TNF-alpha -induced necrosis in tubular epithelium (25). This response is cell type-specific and suggests that pan-caspase inhibition may not be an adequate therapeutic approach to renal injury in which inflammation is contributing the cell death. In tubular cells Fn14 was also required for this necrotic response. The necrotic response was dependent on the generation of oxygen radical species, since the antioxidant BHA afforded protection in tubular cells (Figure 3). We do not yet know inhibition of what specific caspase is important for necrotic cell death to proceed. Since tubular cell injury, including apoptosis and necrosis, is a feature of the most common form of parenchymal acute renal failure (60,61), 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.

7. THE WAY FORWARD

TWEAK regulates a wide range of cellular processes that are involved both in the generation and the recovery from renal injury. Early reports have shown that TWEAK elicits a variety of responses in cultured renal

cells, both from the glomerular and the tubulointerstitial compartments. TWEAK interacts with the cell microenvironment to elicit cell-specific responses. In addition, the expression of both TWEAK and its receptor is increased during acute tubular injury and systemic injection of TWEAK increases the transcription of proinflammatory genes in the kidney. This body of evidence suggests a role for TWEAK in renal injury. Research should focus on the effects of therapeutic interventions aimed at TWEAK, such as soluble Fn14 receptors or neutralizing antibodies on models of glomerular and tubulointerstitial renal injury *in vivo*. This will allow a better definition of the contribution of TWEAK to renal injury and regeneration. Given the pleiotropic actions of TWEAK, that includes modulating the proliferation of progenitor cells (72), agonism of TWEAK should also be explored in certain clinical contexts. In addition, the possible role of TWEAK as a biomarker should be studied as urinary TWEAK levels were found to be elevated in patients with active lupus nephritis (73).

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Abbreviations: TWEAK: Tumor necrosis factor-like weak inducer of apoptosis, mTWEAK: membrane-bound form TWEAK, sTWEAK: soluble form TWEAK, TWEAKR: TWEAK receptor, Fn14: TWEAK receptor factor-inducible 14, TNF: Tumor necrosis factor, TNFSF: Tumor necrosis factor superfamily, TNFR: TNF receptor, TRAF: TNFR-associated factor, PBMC: Peripheral blood mononuclear cells, ECs: Human endothelial cells, SMCs: Smooth muscle cells, INF: Interferon, FGF-1: Fibroblast growth factor-1, NF-kappaB: Nuclear factor-kappaB, ERK: Extracellular signal-regulated kinase, JNK: c-Jun N-terminal kinase, MCP: Monocyte chemotactic protein, RIP: Receptor interacting protein, TRADD: TNFR-associated DD, AKI: Acute kidney injury.

Key Words: Acute Renal Failure, TWEAK, Fn14, Apoptosis, Renal Injury, Tubular Cells, Review

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