1. ABSTRACT

The tumor necrosis factor superfamily (TNFSF) of cytokines comprises 19 ligands and 28 receptors (1). Ligand-mediated activation of TNFSF receptors plays a role in the pathogenesis of multiple central nervous system (CNS) diseases such as multiple sclerosis (2) and cerebral ischemia (3). Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the TNFSF (4) that binds a small cell surface receptor known as fibroblast growth factor-inducible 14 (Fn14) (5-7). There is a growing body of evidence indicating that the interaction between TWEAK and Fn14 plays a role on cell death, regulation of the permeability of the neurovascular unit (NVU) and development of an inflammatory response in the CNS under physiological and pathological conditions (8, 9). Accordingly, here we will review the information available to this date on the role of this cytokine and its receptor in the CNS.

2. INTRODUCTION

2.1. TWEAK and Fn14 structure and signaling pathway

TWEAK is a member of the TNFSF that was initially described as a cytokine with proapoptotic activity on interferon-gamma-treated cells (4). TWEAK is synthesized as a 249-amino acid (aa) type II transmembrane glycoprotein and most cells express both full-length, membrane-anchored TWEAK and a soluble, 156-aa TWEAK extracellular domain fragment (10). TWEAK gene expression has been detected in brain, heart, skeletal muscle and pancreas (10-16) as well as spleen, lymph nodes, thymus, lymphocytes, mouse peritoneal macrophages, fibroblasts, and human smooth muscle cells (4, 17, 18). TWEAK activity is mediated via binding to Fn14 which was initially described as a growth factor-inducible gene encoding a small type I transmembrane protein of unknown function (5), and subsequently...
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3.1. IN THE CNS

3.  BIOLOGICAL ACTIVITY OF TWEAK AND FN14

3.1. NF-kappaB pathway activation

The NF-kappaB family includes five structurally-related proteins that bind to a specific DNA motif and regulate gene expression (29, 30). NF-kappaB functional complexes are found in all cell types in the CNS (31, 32), and can be activated by many different stimuli, including cytokines, via two distinct pathways (29, 30, 33). In the classic or canonical pathway, NF-kappaB complexes are present in the cytoplasm as inactive transcription factors due to their association with the repressor protein IkappaBalpha. Stimulation of cells induces IkappaBalpha phosphorylation, liberation of NF-kappaB, and resultant nuclear translocation and binding to DNA. In the non-canonical pathway, IkappaBalpha is not involved but instead extracellular stimuli induce phosphorylation and proteolytic processing of the NF-kappaB/Rel family member p100, with resultant nuclear translocation of p52 homodimers or RelB/p52 heterodimers. In vitro studies have demonstrated that incubation of EC, and primary neuronal, astrocytic and microglial cultures with TWEAK results in NF-kappaB pathway activation in each cell type (34). In vivo studies have shown that the intracerebral injection of recombinant TWEAK into non-ischemic brains induces IkappaBalpha phosphorylation and nuclear translocation of p65 in neurons and astrocytes (34) (Figure 1). These observations support the hypothesis that TWEAK plays a role in the regulation of the function of the neurovascular unit under physiological conditions.

3.2. Inflammation

Inflammation is a key event in the pathogenesis of several CNS diseases including cerebral ischemia and multiple sclerosis (35) and TWEAK has been shown to be a pro-inflammatory factor in in vitro and in vivo systems (12, 22, 36, 37). Indeed, TWEAK and Fn14 are found in astrocytes and microglial cells (8, 9), suggesting that this cytokine is involved in the genesis of an inflammatory response in the CNS. This hypothesis is supported by the observation that treatment of cultured astrocytes with TWEAK induces a dose-dependent increase in IL-6 and IL-8 secretion as well as in ICAM-1 expression (9). Likewise, TWEAK and Fn14 have been shown to regulate the development of an inflammatory response in an animal model of experimental immune encephalitis (EAE) (9, 38).

3.3. Cell death

The ability of TWEAK to induce cell death in tumor cell lines is relatively weak and in many instances this effect requires incubation with agents such as gamma-interferon (4, 39-41). The mechanism by which TWEAK induces cell death is not completely understood. However, it has been demonstrated that TWEAK may induce cell death through both, TNF-alpha-dependent (42) and –independent (39, 40) pathways. In the CNS, exposure of cultured neurons to TWEAK induces cell death through activation of the NF-kappaB pathway (20). Likewise, inhibition of TWEAK activity with Fn14-Fc decoy following occlusion of the middle cerebral artery results in a significant decrease in apoptotic cell death in the area surrounding the necrotic core (8).

4. TWEAK AND FN14 IN CNS DISEASE

4.1. Ischemic stroke

Ischemic stroke is the third cause of mortality and a leading cause of disability in the United States (43), and the second cause of mortality all over the world (44). It is estimated that in the year 2005 approximately 9.5% of all deaths in the world were due to ischemic stroke or its complications (44). There is a growing body of evidence implicating different cytokines, including TWEAK, in the pathogenesis of cell death and cerebral edema following the onset of the ischemic insult (8, 45). Likewise, it has been demonstrated that inhibition of TWEAK activity early after middle cerebral artery occlusion (MCAO) is protective in at least two different animal models (20, 34), suggesting that the TWEAK-Fn14 axis may be a new therapeutic target for acute cerebral ischemia.

4.1.1. TWEAK and Fn14 expression during cerebral ischemia

TWEAK and Fn14 mRNA increase in the ischemic hemisphere as early as 24 hours after the onset of MCAO (8, 20). This is paralleled by an increase in TWEAK and Fn14 protein expression in the area surrounding the necrotic core (8). Neuroradiological studies have shown that these changes in TWEAK and Fn14 are primarily observed in those areas of the ischemic brain with more than 80% decrease in cerebral blood flow (CBF) but no changes in the apparent diffusion coefficient of water (ADC) signal, demonstrating that the effect of cerebral ischemia on TWEAK and Fn14 occurs mainly in the area of ischemic penumbra (Figure 2; unpublished data).
**Figure 1.** Effect of TWEAK on NF-κB pathway activation in astrocytes, neurons and microglia. Indirect immunofluorescence analysis of p65 subcellular location in murine astrocytes (A-F), neurons (G-L) and microglia (M-R), 1 hour after the intracerebral injection of TWEAK. Frozen sections of brains injected with TWEAK (A-C, G-I and M-O) or PBS (D-F, J-L and P-R). Red is GFAP staining in A & D, Neuronal (NeuN) staining in G & J and MAC-1 staining in M & P. Blue is DAPI. Green is p65 staining in B, E, H, K, M, N and Q. Panels C, F, I, L, O and R represent the corresponding merged images. Thick arrows indicate cells with nuclear translocation of the p65 subunit. Thin arrows indicate the cytoplasmic location of the p65 subunit of the inactive NF-κB complex. Magnification x 100. Reprinted with permission from J Neurosci (34).

4.1.2. Inhibition of TWEAK activity during acute ischemic stroke

Currently there are two protein-based strategies to inhibit TWEAK activity in vivo: a soluble Fn14-Fc fusion protein and anti-TWEAK monoclonal antibodies. The structure and function of the Fn14-Fc fusion protein was first described in 2003 (28). Since then it has been demonstrated that Fn14-Fc inhibits TWEAK biological activity in numerous cell types including mouse astrocytes (9), mouse glomerular mesangial cells (22), mouse Eph4 mammary epithelial cells (24), rat vascular smooth muscle cells (27), and human glioma cells (23, 46). Likewise, anti-TWEAK monoclonal antibodies have been generated in Armenian hamsters using immunization with soluble human TWEAK protein and standard hybridoma generation procedures. The blocking properties of the Fn14-Fc decoy (8) and anti-TWEAK monoclonal antibodies (20) have been tested in a mouse model of cerebral ischemia. Indeed, intraperitoneal administration of anti-TWEAK monoclonal antibodies (20) or intracerebroventricular administration of Fn14-Fc decoy (8) following the onset of the ischemic insult results in a ~30%
Figure 2. Effect of cerebral ischemia on TWEAK-Fn14 expression. Animals were subjected to MCAO followed by continuous MRI monitoring of cerebral blood flow (CBF) and apparent diffusion coefficient (ADC) of water followed by immunohistochemical analysis of TWEAK and Fn14 expression. A. MRI after MCAO. The red area describes the zone of the ischemic cortex irreversible affected (decreased ADC signal). The yellow zone depicts the area of the ischemic cortex with a greater than 80% decrease in CBF but normal ADC signal (ischemic penumbra). B & C. Immunohistochemical staining for TWEAK in the ipsilateral (C) and contralateral (B) hemispheres in the area corresponding to the white square in A. D & E: Immunohistochemical staining with antibodies directed against Fn14 in the area indicated by the white square in A in the ipsilateral (E) and contralateral (D) hemisphere. Magnification X 40.

and ~40% decrease in the volume of the ischemic lesion 48 hours after MCAO, respectively. Furthermore, when compared to littermate controls, Fn14−/− mice exhibit a ~60% in the volume of the ischemic lesion following permanent MCAO (47). Together, these observations support the hypothesis that the interaction between TWEAK and Fn14 has a deleterious effect during cerebral ischemia.

4.1.3. TWEAK and NF-κB pathway activation during cerebral ischemia

NF-κB activity is significantly increased in animal models of ischemic stroke and data obtained from mice deficient in the NF-κB p50 subunit indicates that NF-κB activation enhances ischemic neuronal death (48). Also, NF-κB inhibition using a recombinant adenovirus expressing a dominant negative form of IkappaBalpha reduced ischemic lesion volume in a murine model of cerebral ischemia (49). Several different interleukins and the MMP-9 gene are induced by NF-κB in glia and neurons (31). As mentioned above, TWEAK has been demonstrated to activate the NF-κB pathway in vitro (21, 26-28, 50-52) and in vivo (34) and TWEAK treatment of various cell types has been shown to increase IL-6 and IL-8 production (4, 11, 12, 21, 53, 54). To study the role of TWEAK and Fn14 on cerebral ischemia-induced NF-κB pathway activation, Fn14 deficient (Fn14−/−) and wild-type (WT) mice underwent MCAO. WT mice were treated with either Fc protein (control) or Fn14 Fc decoy immediately after the onset of the ischemic insult. It was observed that compared to control animals, either treatment with Fn14-Fc decoy or genetic deficiency of Fn14 results in a significant decrease in MCAO-induced IKKbeta and p65 phosphorylation (Figure 3) (47). These results demonstrate that the interaction between TWEAK and Fn14 induces activation of the NF-κB pathway during cerebral ischemia.

4.1.4. The TWEAK-Fn14 axis and the permeability of the neurovascular unit

The neurovascular unit (NVU) is a dynamic structure consisting of endothelial cells (EC), the basal lamina, astrocytic end-feet processes, pericytes and neurons (55). The permeability of the NVU is determined not only by the integrity of the interendothelial tight junctions but also by the composition of the basal lamina (55) and the interaction between astrocytes, EC and the extracellular matrix (ECM) (56). It has been reported that proinflammatory cytokines released in response to the ischemic signal act directly on elements of the NVU with resultant increases in BBB permeability (57). To study the effect of TWEAK on the permeability of the NVU, recombinant TWEAK was injected directly into the brain of non-ischemic wild-type mice followed by the intravenous administration of Evans blue dye as a marker of increased blood-brain barrier permeability. It was demonstrated that the injection of TWEAK was associated with a dose-dependent increase in the permeability of the NVU (34), and that this effect was
Figure 3. TWEAK contributes to NF-κB pathway activation following MCAO. Western blot analysis of phospho-IKKβ and total IKKβ (A) and phospho-p65 and total p65 (B), in brain extracts of wild-type mice treated either with Fc protein or Fn14-Fc decoy immediately after MCAO, and Fn14−/− mice, at 0, 1, 3 and 6 hours after the onset of the ischemic insult. Actin expression levels were assayed as a control for protein loading. Each experiment was repeated three times. Graphs describe mean density of the band for a total of 5 observations for each time point. Lines depict SEM. * p< 0.05. Reprinted with permission from J Cereb Blood Flow Metab (47).

not observed when Fn14−/− mice were injected with TWEAK (Figure 4). These results indicate that the interaction between TWEAK and Fn14 has a direct effect on the permeability of the NVU. To further characterize this observation the structure of the NVU in TWEAK- and PBS-injected mice was studied by electron microscopy. These studies showed that the injection of TWEAK induced the development of areas of perivascular edema that were not seen in control animals (Figure 5). All together, these observations demonstrate that TWEAK has a direct effect on the structure and permeability of the NVU under non-ischemic conditions.

To see if the interaction between endogenous TWEAK and Fn14 has an effect on the permeability of the NVU during cerebral ischemia, wild-type and Fn14−/− mice underwent MCAO. Wild-type mice were treated either with Fc protein (control) or Fn14-Fc decoy followed by the intravenous administration of Evans blue dye. Each animal had evaluation of locomotor activity before, and 6, 24 and 48 hours after the procedure. At the end of the last evaluation, brains were extracted and the permeability of the NVU was assessed. These experiments showed that inhibition of TWEAK activity by either treatment with Fn14-Fc decoy or genetic deficiency of Fn14 resulted in a significant amelioration of cerebral ischemia-induced increase in the permeability of the NVU. Furthermore, this protection to the permeability of the NVU was paralleled by a faster recovery of locomotor activity following MCAO (47). In summary, these results indicate that the interaction between TWEAK and Fn14 during cerebral ischemia has a direct effect on the permeability of the NVU and that this effect has a clinical correlation with a slower recovery in locomotor function.

4.1.5. Role of TWEAK in cerebral ischemia-induced MMP-9 activation

Matrix metalloproteinases (MMPs) are matrix-degrading enzymes. In the adult brain, MMP expression is very low. However, in response to a variety of stimuli there is a progressive increase in MMP activity in endothelial cells, astrocytes, microglia and neurons (58). MMP-9 has been directly implicated in the pathogenesis of cerebral ischemia. Indeed, early after the onset of the ischemic insult there is a progressive increase in MMP-9 activity (59, 60), and genetic deficiency of MMP-9 results in a significant decrease in the volume of the ischemic lesion, presumably by preservation of the integrity of the tight junction protein ZO-1 (61, 62).

Several TWEAK-inducible genes, including MMP-9, are known to be regulated via the NF-kappaB pathway (4, 11, 12, 21, 52-54, 63, 64). To study the effect of TWEAK on MMP-9 activity in the CNS, non-ischemic wild-type mice were injected with recombinant TWEAK directly into the brain, and MMP-9 activity was studied by gelatin zymography assay. It was observed that treatment with TWEAK results in a significant increase in MMP-9 activity (34). Furthermore, this effect of TWEAK on MMP-9 was not observed in mice deficient in the p50 subunit of NF-κB (34). These observations support the hypothesis that in the brain TWEAK induces MMP-9 through NF-kappaB pathway activation. Additionally, treatment of cultured astrocytes with TWEAK induces a
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Figure 4. TWEAK increases the permeability of the BBB Evans blue dye extravasation following the intracerebral injection of 2 µl of either PBS or recombinant TWEAK (1 µg/µl) in wild-type (black bars) or Fn14−/− (white bar) mice. Bars denote mean value (n = 6-10) and error bars describe standard deviation of the mean. * p < 0.0001 compared to PBS.

Figure 5. TWEAK disrupts the structure of the NVU. Electron microscopy of cerebral arterioles in the left striatum of mouse brains injected with either PBS (panels A & C) or TWEAK (panels B & D). The asterisks show fluid-fill spaces indicative of developing edema in the TWEAK-treated brain (panels B & D). The arrows indicate places in the neurovascular unit with disruption of the glia limitans and detachment of the astrocytic processes. BV: blood vessel; A: astrocytic processes. Magnification X 5000 in A & B and X 30000 in C & D. Reprinted with permission from J Neurosci (34).

To study the role of TWEAK on MMP-9 activation during cerebral ischemia, wild-type and Fn14−/− mice underwent MCAO followed by treatment with either Fc protein (control) or Fn14-Fc decoy in wild-type mice. Zymography assays performed six hours later demonstrated a significant decrease in MMP-9 activity in wild-type mice treated with Fn14-Fc decoy and in Fn14−/− mice, demonstrating that the interaction between TWEAK and Fn14 plays a significant role in the induction of MMP-9 activity during cerebral ischemia (Figure 6)(47). Additionally, the effect of Fn14-Fc decoy on MMP-9 activity during MCAO was shown to be dose-dependent (47).

4.1.6. TWEAK and the basement membrane
The composition of the basement membrane and the interaction between perivascular astrocytes and the basal lamina are important determinants of the integrity and function of the NVU (55, 65). Indeed, early after the onset of cerebral ischemia there is a progressive degradation of components of the basal lamina and detachment of astrocytes from the underlying basement membrane (65). To study the role of TWEAK on the integrity of the interaction between perivascular astrocytes and the basement membrane, non-ischemic mice were injected with recombinant TWEAK directly into the brain, followed 24 hours later by electron microscopy analysis of the architecture of the NVU. It was observed that treatment with recombinant TWEAK results in edema of astrocytic end-feet processes, with disruption of the glia limitans and detachment from the basement membrane (34).

Laminin is one of the most abundant components of the basal lamina and a substrate for MMP-9 (66). To study the effect of TWEAK on laminin degradation following cerebral ischemia, wild-type and Fn14−/− mice underwent MCAO followed by treatment with Fc protein (control) or Fn14-Fc decoy in wild-type animals. Laminin degradation was assessed by immunoblotting and immunohistochemistry 24 hours later. It was found that inhibition of TWEAK activity by either Fn14-Fc decoy or genetic deficiency of Fn14 results in a significant decrease in cerebral ischemia-induced laminin degradation (47).

Together, these results allow us to propose a model where in response to the ischemic insult there is an increase in TWEAK activity. This TWEAK then interacts with Fn14 in the NVU. This interaction results in activation of the NF-κB pathway and release of proinflammatory cytokines and matrix metalloproteases known to have a direct effect on the structure of the NVU and the permeability of the BBB.

4.2. Multiple sclerosis
Multiple sclerosis is a chronic progressive disease characterized by loss of myelin in the CNS with subsequent neuronal damage (67, 68). Experimental autoimmune encephalomyelitis (EAE) is a research model for multiple sclerosis (69). TWEAK mRNA increases in the spinal cord during EAE and the clinical severity of EAE is significantly enhanced in TWEAK-overexpressing transgenic mice (9). Additionally, TWEAK stimulates monocyte chemotactic protein-1 (MCP-1) expression by astrocytes and EC (63), and induction of specific inhibitory antibodies by treatment with either TWEAK or Fn14 results in amelioration of the development of inflammatory...
infiltrates in the spinal cord and in a better clinical outcome in a rat model of EAE (38). A potential application for inhibition of TWEAK activity in multiple sclerosis was further supported by the demonstration that treatment with neutralizing anti-TWEAK antibodies in a model of EAE results in a reduction in the severity of the disease and leukocyte infiltration when mice were treated after the priming phase (70).

5. SUMMARY

In this review we have summarized the information available to this date indicating that TWEAK and Fn14 are expressed in the NVU and that the interaction between this cytokine and its receptor plays a role in the regulation of the permeability of the blood-brain barrier, cell death and the development of an inflammatory response in the CNS. The interaction between TWEAK and Fn14 induces activation of the NF-kappaB pathway in EC, astrocytes, neurons and microglia with resultant up-regulation of NF-kappaB-regulated genes. There is evidence that under ischemic and non-ischemic conditions TWEAK induces the activation of MMP-9, an NF-kappaB-dependent gene with known effects on the architecture and permeability of the NVU, as well as cell death.

There is a growing body of evidence indicating that the interaction between TWEAK and Fn14 plays a significant role in the sequence of events leading to cerebral edema and cell death during ischemic stroke, as well as the infiltration of inflammatory cells during EAE. Accordingly, inhibition of TWEAK activity with either an Fn14-Fc fusion protein or anti-TWEAK monoclonal antibodies has demonstrated to be protective during cerebral ischemia and an animal model of multiple sclerosis, suggesting that TWEAK is a molecular target for the treatment of these diseases.

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7. REFERENCES


