Published in final edited form as:

Prog Neurobiol. 2007 December ; 83(6): 363–374.

Tumor necrosis factor and stroke: role of the blood-brain barrier

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Abstract

The progression and outcome of stroke is affected by the intricate relationship between the blood-brain barrier (BBB) and tumor necrosis factor α (TNF α). TNF α crosses the intact BBB by a receptor-mediated transport system that is upregulated by CNS trauma and inflammation. In this review, we discuss intracellular trafficking and transcytosis of TNF α , regulation of TNF α transport after stroke, and the effects of TNF α on stroke preconditioning. TNF α can activate cytoprotective pathways by pretreatment or persistent exposure to low doses. This explains the paradoxical observation that transport of this proinflammatory cytokine improves the survival and function of hypoxic cells and of mice with stroke. The dual effects of TNF α may be related to differential regulation of TNF α trafficking downstream to TNFR1 and TNFR2 receptors. As we better understand how peripheral TNF α affects its own transport and modulates neuroregeneration, we may be in a better position to pharmacologically manipulate its regulatory transport system to treat stroke.

Keywords

TNF α ; stroke; blood-brain barrier; transcytosis; transport; hypoxia; ischemia; preconditioning

1. Introduction

1.1. The blood-brain barrier (BBB)

The BBB is a large interface between the brain and its supplying blood vessels. Its main effective site consists of exchange vessels with thin walls and large surface areas, including capillaries and post-capillary venules. These microvascular endothelial cells are joined by tight junctions and fortified by a continuous basement membrane and astrocytic endfeet on the basolateral side. Pericytes and extracellular matrix also participate in the formation of this multi-dimensional “neurovascular unit”. The surface area of the BBB is 100 to 150 cm²/g, by contrast to the circumventricular organs that have a total surface area of only 0.02 cm²/g tissue (Strand, 1999; Begley, 1994). This vast interface regulates the controlled permeation of ions, sugars, nucleosides, amino acids, peptides, and proteins (Johanson, 1995; Zlokovic, 1995; Peruzzo *et al.*, 2000; Bickel *et al.*, 2001; Abbruscato *et al.*, 2004). The BBB also plays an essential role in provision of neurotrophic support and remodeling of the extracellular matrix (Pan *et al.*, 1998; Lo *et al.*, 2002). During the past two decades, specific transport systems for selective proinflammatory cytokines and neurotrophic proteins have also been identified at the BBB (Banks *et al.*, 1991; Pan and Kastin, 2003).

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1.2. Stroke affects the BBB and cytokine transport

Stroke is the third most common cause of death after heart attack and cancer and has profound negative social and economic effects. The current treatment for complete stroke has limited success in reversing neurodegeneration and restoring premorbid function. Although it is common wisdom that stroke disrupts the BBB, it is not widely realized that the altered BBB in turn affects stroke progression and neuroregeneration. A general focus of this review, therefore, is on the transport systems for cytokines which are key modulators of inflammation and regeneration. The specific focus is on the mechanisms of regulation of the transport of the cytokine tumor necrosis factor α (TNF α) across the BBB as it affects recovery after stroke.

The interaction of TNF α with the BBB can be determined both in the intact organism and in cultured cells. The goals of transport studies include identification of the links between TNF α permeation and ischemic tolerance, determination of new cellular targets for cytoprotection, and development of novel strategies for better treatment of stroke victims. *In vitro*, cerebral microvessel endothelial cells can be isolated and subjected to hypoxic and hypoglycemic conditions commonly seen after stroke. Imaging techniques to determine cerebral blood flow, metabolism, and BBB permeability can be used in patients after stroke. Animal models of stroke also are well established. The most commonly used model of transient middle cerebral artery occlusion (tMCAO) with reperfusion accurately reflects the human disease of thromboembolic stroke.

It should be pointed out that the current animal models of stroke mainly involve ischemic lesions resulting from large vessel occlusion, although small vessel diseases are more common in clinical settings. Occlusion of small vessels can occur with hypertension, diabetes, cigarette smoking, hyperlipidemia, and abuse of certain drugs, all being risk factors for stroke. Vessel wall damage takes place in atherosclerosis, hyalinosis, amyloid angiopathy, and BBB dysfunction, and cerebrovascular insufficiency can be caused by disturbances of systemic circulation, perfusion vulnerability related to the vascular anatomy of the brain, disturbance of autoregulation, and hyperviscosity. All of these contribute to multi-infarct dementia (Wallin and Blennow, 1993). Intracarotid injection of multiple microemboli has been used to simulate the more diffuse small vessel ischemic stroke (Overgaard *et al.*, 1992; Rapp *et al.*, 2003; Atochin *et al.*, 2004), but filament occlusion or surgical ligation of the middle cerebral artery is much more commonly used. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) involves mutation of the NOTCH3 gene and affects mainly vascular smooth muscle cells; transgenic mouse models have been useful to address the pathophysiology (Fryxell *et al.*, 2001; Lacombe *et al.*, 2005). Overall, there remains a gap between effective studies in a variety of animal models and clinical success with new experimental drugs (Dirnagl *et al.*, 1999).

2. TNF α in stroke

2.1. Regulation of TNF α expression in the CNS after stroke

One of the first indications that TNF α is an important mediator of stroke is the correlation of its expression with stroke damage. Real-time PCR analysis in rats after tMCAO shows that TNF α mRNA is increased 3 h after stroke and persists for 24 h in the hemisphere ipsilateral to occlusion, accompanying changes in mRNA for interleukin (IL)-1 β , IL -6, E-selectin, and intercellular adhesion molecule-1 (ICAM-1). Meanwhile there is increased immunoreactivity for activated nuclear factor (NF) κ B, and infiltration of inflammatory cells (Berti *et al.*, 2002). TNF α protein also is increased in cerebral tissue 6 and 24 h after MCAO (Haddad *et al.*, 2006). After permanent MCAO, TNF α expression increases not only in neurons, but also in astrocytes, microglia, choroid plexus, endothelial cells, and infiltrating polymorphonuclear

leukocytes. Pericytes in the rat lung secrete TNF α when stimulated by lipopolysaccharide (Edelman *et al.*, 2007), but it is not certain whether pericytes at the BBB also produce TNF α .

TNF α produced by brain parenchymal cells may be beneficial for stroke recovery at certain time points. Astrocytes show robust TNF α immunoreactivity at day 17 – 18, lagging behind neuronal expression which peaks 2 – 3 days after human stroke. This may suggest a role of TNF α in tissue regeneration (Sairanen *et al.*, 2001). Further, the level of TNF α in the CSF of stroke patients correlates with anti-apoptotic bcl-2 expression, indicating that TNF α may not be entirely detrimental to recovery (Tarkowski *et al.*, 1997).

2.2. Circulating TNF α concentrations after stroke

How important is circulating TNF α in the overall concentration of TNF α in the ischemic brain? What are its cellular sources? In clinical studies, it has been shown that elevated blood concentrations of TNF α cannot be completely explained by leakiness or efflux of TNF α after cerebral production. Increased serum and CSF levels of TNF α have been found in patients 24 h, 1 week, and 2 weeks after stroke, and this correlates with infarct volume and severity of neurological impairment. Regardless, disruption of the BBB indicated by the CSF/serum albumin ratio is minimal in most patients (Zaremba and Losy, 2001). This suggests an important role of inflammatory cells in the microcirculation as a source of TNF α in the periphery. More definitively, Ferrarese *et al.* showed a three-fold increase of TNF α from stimulated blood cells 1 – 90 days after stroke (Ferrarese *et al.*, 1999). Increased production of proinflammatory cytokines in the microcirculation outside the BBB after ischemia and inflammation is somewhat analogous to the lung inflammation occurring in acute asthma. In this condition, permeability of the blood-alveolar barrier is increased and blood-borne chemokines may reflect the extent of inflammation (Quinton *et al.*, 2004).

Peripheral production of TNF α is seen not only in clinical cases but also in animal studies. At 6 and 22 h after MCAO, activated spleen cells are a major source of secretion of TNF α and some other cytokines (Offner *et al.*, 2006). This is associated with marked proinflammatory changes in the brain. Thus, the neuroimmune axis appears to have a feedback loop, in that focal cerebral ischemia results in widespread activation of inflammatory cytokines in peripheral immune organs, and this in turn modulates CNS pathophysiology.

2.3. Dual roles of TNF α

Both injurious and beneficial roles of TNF α have been proposed for TNF α in the pathogenesis of cerebral ischemia (Meistrell *et al.*, 1997; Pan *et al.*, 1997c; Dziewulska and Mossakowski, 2003). On the one hand, blockade of TNF α actions reduces infarct volume after permanent MCAO, as seen in BALB/C mice with the dimeric type I soluble TNF receptor which binds to TNF α and antagonizes its action (Nawashiro *et al.*, 1997a). Similarly, the anti-TNF α antibody PI14 and the synthesis inhibitor CNI-1493 also improve the deficits in Lewis rats after stroke (Meistrell *et al.*, 1997). Treatment with the poly(ADP-ribose) polymerase inhibitor PJ 34 (Haddad *et al.*, 2006), the proteasome inhibitor MLN519 (Williams *et al.*, 2004), or the tree-derived dye brazilien (Shen *et al.*, 2007) is associated with reduced brain TNF α expression after tMCAO. All these experimental manipulations reduce the area of infarct and neurological deficits; this indicates a deleterious role of TNF α in stroke progression in these animal models.

On the other hand, TNF α pretreatment is neuroprotective against permanent MCAO in BALB/C mice with reduction of infarct size, CD11b-positive neutrophils, and macrophages (Nawashiro *et al.*, 1997b). Knockout mice deficient in TNF α receptors have enhanced sensitivity to stroke, with exacerbated neuronal damage (Bruce *et al.*, 1996). TNF α can also mediate neuroprotection in other situations. In one recent study, sodium nitroprusside was used to induce acute nitric oxide excitotoxicity in TNF α knockout mice. These mice showed

dramatic exacerbation of neuronal damage, suggesting that early endogenous TNF α release after the insult is neuroprotective (Turrin and Rivest, 2006). In another study, TNF α -expressing neurons from TNF α -transgenic mice were strongly protected from apoptosis induced by glutamate, a substance inducing excitotoxicity in primary cortical neurons. Neurons from wild type mice pretreated with TNF α were also resistant to excitotoxicity (Marchetti *et al.*, 2004). Further, excitotoxic neuronal death induced by N-methyl-D-aspartate (NMDA) is reduced by TNF α treatment in cultured cortical neurons, and this is mediated by TNFR1 (Carlson *et al.*, 1998).

Thus, several variables probably contribute to the different actions of TNF α , as different types of studies presumably involve the activation of different cellular targets, with time of administration being another factor. Differences in the route of administration might also contribute to the paradoxical effects, as TNF α blocking agents are frequently administered peripherally whereas TNF α pretreatment is generally given intracisternally. While findings from animal studies are helpful in guiding human stroke trials, the events and mechanisms in human stroke are not identical to those in animal models. Analogous to stroke, anti-TNF α treatment reduces demyelinating lesions in experimental autoimmune encephalomyelitis (Selmaj *et al.*, 1991), but fails to show clinical efficacy in patients with multiple sclerosis, in contrast to the significant improvement seen after anti-interferon γ antibody (Skurkovich *et al.*, 2001).

3. TNF receptors and their regulations after stroke and by TNF α

3.1. Apoptotic and cytoprotective pathways

TNFR1 (p55 or p60) and TNFR2 (p75 or p80) are type-I cytokine receptors that mediate distinct biological functions of TNF α . TNFR1 contains a death domain (DD) in the cytoplasmic tail that is not present in TNFR2. The signaling of TNFR1 is better elucidated and has been recently reviewed extensively elsewhere (Beere, 2005; Hehlhans and Pfeffer, 2005; van Horssen *et al.*, 2006). Briefly, TNFR1 has three major cytoplasmic domains: the TNFR1 internalization domain (TRID), neutral sphingomyelinase domain (NSD), and the DD (Neumeyer *et al.*, 2006). TRID is essential for receptor internalization and initiation of apoptosis. NSD is the site where the factor associated with neutral sphingomyelinase (FAN) interacts with the receptor, which leads to production of ceramide and activation of caspase-3 (Adam-Klages *et al.*, 1996). DD is the binding site for TNFR-associated death domain (TRADD), which recruits adaptor proteins receptor interacting protein (RIP), TNFR-associated factor 2 (TRAF-2), and Fas-associated death domain (FADD). From here, the signal pathways diverge: association of FADD with pro-caspase-8 leads to apoptosis, whereas association of TRAF-2 with the cytoplasmic inhibitor of apoptosis protein (cIAP) signals survival by transcriptional activation of NF κ B and cFos/cJun (Fig. 1). The molecular switches and dynamic changes in signaling elements are pertinent to the multi-faceted roles of TNF α in stroke progression and rehabilitation.

3.2. Differential distribution of receptor subtypes

TNF α receptors in the brain exhibit regional specificity. TNF α binding is highest in the mouse brainstem and lowest in the cerebellum (Kinouchi *et al.*, 1991). The receptor densities and dissociation constants of ¹²⁵I-human TNF α have been characterized in multiple lines of cells originating in sites outside the CNS (Yoshie *et al.*, 1986; Pennica *et al.*, 1992). The two TNF α receptors have different localization within the same cell. In human umbilical vein endothelial cells, TNFR2 is mainly present at the cell surface whereas TNFR1 is predominantly localized to the Golgi apparatus and is less prevalent at the cell surface and in cytoplasmic vacuoles (Bradley *et al.*, 1995). The distinct intracellular localization of the two receptors could be important in the transport of TNF α as well as in signal transduction. Figure 2 proposes

alternative models of transcytosis of TNF α based on the differential distribution of the receptor subtypes. Ligand-passing during the course of intracellular trafficking has been suggested for the axonal transport of neurotrophin-3 (Butowt and von Bartheld, 2001; Wang *et al.*, 2002).

The divergent TNF α signaling pathways, particularly those leading to cytotoxicity and NF κ B activation, have been studied extensively. By contrast, trafficking of TNF α inside the cell has been less well studied, as protein ligands were formerly thought to undergo complete degradation after activation of membrane receptors. Nonetheless, it is certain that TRID-mediated internalization of TNFR1 is essential for the formation of the death-inducing signaling complex which leads to the caspase cascade and apoptosis (Schütze *et al.*, 1999; Schneider-Brachert *et al.*, 2004; Neumeyer *et al.*, 2006).

3.3. TNF α and its receptors after stroke

After permanent MCAO in rats, upregulation of TNFR1 is present at 6 h and precedes upregulation of TNFR2, which occurs at 24 h (Botchkina *et al.*, 1997). *In vitro*, pretreatment with TNF α reduces expression of TNF α receptors under hypoxic/reoxygenated conditions, and this reduced receptor expression may be related to a reduction in brain damage observed 2 h after MCAO (Ding *et al.*, 2006).

These contrasting actions of TNF α in stroke are further illustrated in other types of studies. Mice lacking TNFR1 show a larger infarct area than wild type or TNFR2 knockout mice after MCAO, and sustain more damage of CA3 hippocampal neurons by kainic acid excitotoxicity (Gary *et al.*, 1998). By contrast, TNFR2 signaling is implicated in endothelial activation and inflammatory ischemia (Akassoglou *et al.*, 2003). In some neurodegenerative processes, however, TNFR2 can exert neuroprotective effects. Marchetti *et al.* found that neurons from TNFR2 knockout mice are susceptible to glutamate-induced cell death, whereas wild type and TNFR1 knockout mice are protected against the excitotoxicity (Marchetti *et al.*, 2004). This neuroprotective effect of TNFR2 is mediated through a TNFR2-PI3K-Akt-NF- κ B pathway.

4. Interactions of TNF α with the BBB

4.1. BBB transport of TNF α

Peripheral TNF α has been shown to exert multiple neuroendocrine and immunomodulatory effects, many of them mediated by the central nervous system (CNS) (Pan *et al.*, 1997c). Besides the complete passage of intact radioactively labeled TNF α from blood to brain and cerebrospinal fluid (CSF) in mice (Gutierrez *et al.*, 1993; Pan *et al.*, 1997b), this also has been shown in monolayers of cultured cerebral microvessel endothelial cells (Pan *et al.*, 2003a).

TNF α is one of the cytokines that can cross the BBB by receptor-mediated endocytosis (Gutierrez *et al.*, 1993; Pan and Kastin, 2002; Pan *et al.*, 2003a). Transport is upregulated under conditions that appear to coincide with recovery of the mouse after CNS trauma, inflammation, autoimmune disorder, global ischemia, and pathological aging (Pan *et al.*, 1997a; 1999; 2003a; 2003b; Pan and Kastin, 2001; Banks *et al.*, 2001; Osburg *et al.*, 2002). The unique temporal and spatial patterns indicate that TNF transport is mediated by tissue and soluble factors.

The beneficial or deleterious roles of TNF α are further illustrated by its exogenous administration as well as genetic models, including knockout and transgenic mice with mutations of TNF α or its receptors (Pan *et al.*, 1997c). This will be further discussed in the following sections. In a broader sense, regulatory changes of the transport system indicate that the BBB is intimately involved in communication between peripheral cytokines and the CNS, and suggest its essential role in CNS regeneration. Pharmacologically, such regulatory

functions should be considered in the design of a new class of therapeutic agents in conditions such as stroke.

4.2. Role of TNF α receptors in BBB transport of TNF α

The use of knockout mice lacking TNFR1, TNFR2, or both receptors shows that both TNFR1 and TNFR2 are involved in the transport of TNF α across the normal BBB. In the absence of only one of the receptors, the permeability of TNF α from blood to brain is significantly reduced but not absent. Double receptor knockout, by contrast, completely abolishes the transport (Pan and Kastin, 2002). This is consistent with findings in Transwell cultured primary cerebral microvessel endothelial cells from the double receptor knockout mice, which show a significant reduction of the apical-to-basolateral flux compared with cells from the control mouse brain (Pan *et al.*, 2003a). In a rat study, TNFR2 appears to have a greater role than TNFR1 in the transport of TNF α . The mRNA for TNFR2 shows a smaller increase than TNFR1 after intraperitoneal challenge with the endotoxin lipopolysaccharide (Osburg *et al.*, 2002).

Receptor overexpression studies with HEK293 cells present further evidence of the differential involvement of TNFR1 and TNFR2 in the surface binding, endocytosis, and exocytosis of TNF α . Although this is not a BBB cell line, which limits the interpretation of certain aspects of the trafficking results, HEK293 cells provide a high level of overexpression that facilitates the testing of potential cooperative interactions. TNFR2 has a higher capacity than TNFR1 for cell surface binding, whereas TNFR1 induce faster endocytosis. At any time point, there is a greater percent exocytosis of intact TNF α by TNFR2 than by TNFR1 (Pan *et al.*, 2007b). Rather than being degraded, most TNF α inside the cell remains intact for at least 1 h, showing that both receptors can exert protective roles against degradation. Interestingly, the spatial difference of the receptor subtypes does not lead to synergistic actions in the receptor-mediated trafficking of TNF α . More interestingly, TNF α associated with TNFR1 showed less degradation, revealed when the chromatographic data was plotted to normalize the intact values in the exocytosis medium so that the degraded material adjusted proportionally (Fig. 3).

The eventual dissociation of the TNF α -receptor complex remains to be investigated. Some progress with the imaging approach has been made by use of low molecular weight tags, including ^{125}I and biotin. After intravenous injection of ^{125}I -TNF α into mice, electron microscopic autoradiography of ultrathin sections of the brain shows passage into the brain parenchyma. In cultured RBE4 cerebral microvessel endothelial cell line, endocytosed biotinylated TNF α was detected by Quantum dots conjugated with streptavidin, and its time-dependent colocalization with intracellular organelles was shown by confocal microscopy to avoid the usual degradative pathways (Pan *et al.*, 2007a). These new imaging approaches, combined with *in vitro* transport assays, HPLC, and overexpression studies, show vesicular trafficking of TNF α leading to substantial exocytosis of the cytokine in intact form.

4.3. TNF α affects BBB permeability

Interactions of TNF α with cerebral endothelial cells affect not only TNF α transport but also the paracellular permeability of the BBB. TNF α -mediated changes include alterations in cytoskeletal organization and tight junction protein expression as well as production of serine proteases involved in BBB disruption, tissue remodeling, and neural plasticity (Stolphen *et al.*, 1986; Dobbie *et al.*, 1999; Rosenberg, 2002). TNF α also induces cerebral endothelial cell expression of genes involved in such processes as cell adhesion, chemotaxis, apoptosis, transcriptional regulation, and neuroprotection (Franzén *et al.*, 2003).

TNF α increases the transcytosis of holotransferrin in cerebral microvessel endothelial cells co-cultured with glial cells (Miller *et al.*, 2005). This involves the activation of p42-44 mitogen activated protein kinase (MAPK), as co-treatment with U0126 inhibits the transcytosis.

TNF α also increases cellular permeability to fluorescein-labeled dextran (3 kD), and this involves activation of the NF κ B pathway (Trickler *et al.*, 2005).

5. Intracellular trafficking of TNF α receptors

5.1. Lessons from non-BBB cells about TNFR trafficking and ligand endocytosis

The actions mediated by TNFR1 are better characterized than the biological effects of TNFR2. Both NSD and DD in the cytoplasmic tail of TNFR1 play essential roles in recruiting a cell type specific signaling complex (Hallenbeck, 2002). In non-BBB cells, there have been several studies of receptor-mediated endocytosis of TNF α (Mosselmans *et al.*, 1988; Higuchi and Aggarwal, 1994; Shurety *et al.*, 2001), receptor internalization (Ding *et al.*, 1989; Bradley *et al.*, 1993; Schütze *et al.*, 1999), and receptor recycling (Vuk-Pavlovic and Kovach, 1989). TNF α receptors can be internalized by clathrin-mediated endocytosis and trigger signaling upon endocytosis. There are 3000 – 6000 TNF α binding sites in an L929 cell. In these cells, colloid gold conjugated TNF α is rapidly internalized in clathrin-coated vesicles and targeted to endosomes and multivesicular bodies. The signal eventually appears in lysosomes after 30 min (Mosselmans *et al.*, 1988). This suggests that ligand-receptor dissociation probably occurs in the lysosomes; however, the extent of protein degradation was not measured in this study. Nonetheless, there are sorting motifs in TNF receptors. TNFR1 internalization can be mediated by the YXXW motif, located at the membrane juxtapositional cytoplasmic domain (Schneider-Brachert *et al.*, 2004). It is not clear whether polarized sorting of the receptors in the endothelial cells composing the BBB entails a greater percentage of transcytosis of TNF α in intact form, whereas parenchymal cells show a higher extent of intracellular degradation. It is also uncertain whether clathrin, caveolin, or non-clathrin, non-caveolin pathways mediate the differential fate of TNF α inside the cells. Further comparative studies will be helpful to draw clearer conclusions.

These findings from other cell types could provide a basis for identification of the sorting signals at the BBB. In endothelial cells, however, particularly of CNS origin reflecting BBB function, the distribution of receptor subtypes and composition of signaling complexes remain largely unexplored, as do the corresponding patterns of transcytosis and cell death/survival functions.

5.2. Other putative transport-associated proteins

Certain adaptor and other substances are involved in both signaling and trafficking. Examples include Rab5, Cbl, hepatocyte growth factor regulated tyrosine kinase substrate downstream to the epidermal growth factor receptor, PI3K, MAPK, lipids, and calcium influx in insulin receptor trafficking (Sasaoka *et al.*, 1999; Clague and Urbé, 2001; Burke *et al.*, 2001; Predescu *et al.*, 2001; Wang *et al.*, 2002). For TNF α , TRAFs play important roles by interacting with both receptors and downstream pathways. TRAF2, or sorting nexin 6, have essential trafficking functions (Parks *et al.*, 2001). Activation of MAPK p42^{mapk/erk2}, downstream to TRAF2, also phosphorylates TNFR1 and leads to relocalization of TNFR1 from the Golgi apparatus to the endoplasmic reticulum (Cottin *et al.*, 1999). It remains to be determined how TRAF activation affects TNF α trafficking across the BBB.

5.3. TNF signaling affects trafficking of other proinflammatory cytokines

In many instances of inflammation and immune reaction, elevated concentrations of TNF α at the luminal surface of microvessels are not isolated events. How does TNF α , one of the early proinflammatory signals, modulate the cascade of reactions being translated into a CNS response? We used regulation of the cellular response to another proinflammatory cytokine - leukemia inhibitory factor (LIF) - as a model to test signal amplification across the BBB.

LIF binds to type-I cytokine receptors that differ in structure and signaling from TNFRs; these are the specific gp190 (LIFR) and the co-receptor gp130 shared with other members of the IL-6 family of cytokines. In RBE4 immortalized rat cerebral microvessel endothelial cells, both receptors show high levels of protein expression, but they respond to TNF α treatment in opposite directions. The gp190 receptor is down-regulated in a dose- and time-dependent manner (Yu *et al.*, 2007b), whereas the gp130 receptor is upregulated (Yu *et al.*, 2007c). The opposing actions of TNF α on gp190 and gp130 receptors for LIF are illustrated in figure 4. The effects are specific, not caused by cytotoxicity, as evidenced by γ -glutamyl transpeptidase (γ GT) and cellular ATP production (Yu *et al.*, 2007a). Furthermore, activation of NF κ B, which may be mediated by TNF α , does not participate in lysosomal degradation of gp190 but is involved in upregulation of gp130. The co-receptor, or affinity converter gp130, is subject to transcriptional regulation by TNF α in response to NF κ B. The net effect of TNF α is reduced Stat3 activation and reduced transport of LIF (Yu *et al.*, 2007c). This might be extrapolated to represent an adaptive response of the endothelial cells composing the BBB, in that activation of TNF α signaling may restrict further permeation of LIF from blood to CNS.

6. TNF α transport in mice after stroke

6.1. Other molecules induced by stroke that may interact with TNF α

Stroke not only elevates focal and blood concentrations of selective cytokines and chemokines, but it also induces the expression of extracellular matrix proteins and other molecules. Among the potential general markers for stroke severity and progression are selectins and other adhesion molecules, plasminogen activator inhibitor, secretory phospholipase A(2) IIA, phosphatidylcholine, disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-1 and ADAMTS-4 (Cross *et al.*, 2006; Licata *et al.*, 2006; Adibhatla and Hatcher, 2007).

Within the microvessel endothelial cells, which are the main constituent of the BBB, γ GT is an enzyme enriched at the apical surface of endothelial cells and a reliable marker for the BBB (Frey *et al.*, 1991; Roux *et al.*, 1994; Wolf and Gassen, 1997). The activity of γ GT can be altered by several conditions, including inflammation and oxidative stress (Singh *et al.*, 1986; Kugelman *et al.*, 1994). We measured γ GT activity after tMCAO in mice since it is an indicator of endothelial malfunction and BBB damage (Yu *et al.*, 2007a). At the earlier times (12 h to 5 d) after stroke, γ GT levels are increased, but at later times (10 to 15 days) after stroke, they are decreased. In RBE4 cells, γ GT activity is increased by treatment of TNF α and lipopolysaccharide, indicating the stimulatory effects of inflammatory signals. Thus, the later reduction seen in stroke mice is probably related to tissue damage after stroke (Yu *et al.*, 2007a). Therefore, γ GT can be a sensitive indicator of the dynamic changes of endothelial function after stroke.

6.2. Regulation of the TNF α transport system in mice after stroke

A mouse model of tMCAO was used to investigate how the TNF α transport system at the BBB participates in stroke progression and recovery (Pan *et al.*, 2006). After 1 h occlusion followed by nearly complete reperfusion, the neurological deficit lasts more than a week. Even in the presence of a prominent infarct area, the volume of distribution of the vascular marker albumin is unchanged over the 2-week observation period. By contrast, the transport of TNF α is significantly increased 5 d after tMCAO. Since TNF α circulates as a trimer (Smith and Baglioni, 1987), its size is comparable to that of albumin. Although studies with smaller permeability markers such as sodium fluorescein have shown BBB disruption after hypoxia (Schoch *et al.*, 2002), the latent increase of TNF α uptake in the tMCAO mice is apparently not caused by leakage through disruption of the BBB. The increased transport of TNF α is abolished by excess TNF α , indicating both persistence of the saturable transport system for TNF α and

its ability to function at a higher capacity. Striking upregulation of mRNA expression for TNFR1 (15-fold) and TNFR2 (30-fold) was found in the ischemic hemisphere 5 to 7 d after tMCAO, partially explaining the increased transport. However, increased TNF α transport occurs bilaterally, suggesting a role for global mediators in addition to the transporting receptors. If so, remodeling of CNS regions has broader consequences than expected. Above all, upregulation of the TNF α transport system at the BBB after stroke may facilitate neuroplasticity and play an important role in stroke recovery.

7. Studies with hypoxia and altitude sickness

Global hypoxia with eventual reduction of cerebral blood flow and metabolism is seen in a variety of cardiac and pulmonary disorders, including heart attack, uncompensated respiratory acidosis, carbon dioxide poisoning, and obstructive sleep apnea syndrome. TNF α has been proposed to be a mediator in hypoxic and ischemic preconditioning with reduced mortality rate in elderly patients with sleep apnea (Lavie and Lavie, 2006). Altitude sickness and adaptation provide a good model to study how TNF participates in hypoxic conditioning. Oxygen tension is about 16% at 2 km above sea level and 10.8% at 5 km (Xu *et al.*, 2005). To put this into perspective, the highest elevation in the world is Mount Everest of the central Himalaya Mountains at the border between Tibet and Nepal, with a height of 8853.5 m (29,028 ft) and O₂ tension of less than 8%. While regulation of the autonomic nervous system plays a major role in individual responses, cytokine signaling may be critically involved. One of the key signaling elements is TNF α -induced NF κ B activation (Jones *et al.*, 2005; Lee and Hallenbeck, 2006).

High altitude pulmonary edema is associated with a transient increase of TNF α and IL-8 concentrations in bronchoalveolar lavage fluid (Kubo *et al.*, 1996). Prolonged inflammation is a poor prognostic factor for acute respiratory stress syndrome, suggesting an inverse causal relationship between vascular permeability and recovery process. The active transport of selective chemokines in the lungs may also play an important role (Quinton *et al.*, 2004). Impairment of the BBB and neuronal damage in acute mountain sickness may be secondary to pulmonary compromise, but TNF α plays a role that appears to be detrimental (Bailey *et al.*, 2004). It has not been established, however, whether TNF α participates in chronic adaptation to high altitudes.

8. Ischemic tolerance (preconditioning) and TNF α pretreatment

8.1. Preconditioning

Not only is TNF α crucially involved in the initiation, progression, and regeneration of stroke, it also participates in the development of tolerance to ischemia (Masada *et al.*, 2001; Hallenbeck, 2002). Ischemic/hypoxic tolerance, or preconditioning, is an adaptive mechanism in many organs and species whereby mild ischemia and hypoxia can induce neuroprotection against the more severe injury that occurs subsequently. Preconditioning is seen in a variety of pathophysiological conditions, including inflammation, heatstroke, cerebral hemorrhage, transplantation, cell immunity (graft rejection), metabolic disturbance, epilepsy, severe stress, and ischemia of many organs. At least in the brain, a low level of transcriptional activation of TNF α is seen in the ipsilateral cortex 1 h – 2 d after preconditioning, induced by 10 min of MCAO in the rat (Wang *et al.*, 2000).

Cerebral ischemic tolerance after 15 min of preconditioning can change the expression of different classes of genes involved in the stress response, metabolism, transport, cytoskeleton, cell cycle regulation, and signal transduction. Even though injurious ischemia upregulates the expression of multiple genes, preconditioning before the injurious ischemia results in pronounced downregulation of some of the genes. TNFR1 mRNA is increased 72 h after

preconditioning as well as 24 – 72 h after injurious ischemia. However, the combination of both abolishes this increase (Stenzel-Poore *et al.*, 2003). When preconditioning by 10 min of tMCAO reduces the volume of infarct induced by permanent MCAO, there is concurrent upregulation of neuronal TNFR1 expression (Pradillo *et al.*, 2005). TNFR1-antisense oligodeoxynucleotide reduces TNFR1 after intracerebral administration and also decreases the protective effect of the ischemic preconditioning on the size of the infarct. This suggests a protective role of TNFR1 in ischemic tolerance.

Re-programming of the cerebral response to ischemia by preconditioning is illustrated by induction of chaperon proteins, including heat shock protein 70 (Hsp70). Hsp70 correlates with attenuation of brain edema, BBB disruption, and vascular injury after permanent MCAO (Masada *et al.*, 2001). It is now known that the mechanisms of ischemic tolerance involve complex and redundant signaling cascades, well discussed by Dirnagl *et al.* (Dirnagl *et al.*, 2003). The induction can be rapid or delayed for hours and days. The execution of cell protective pathways entails sequential changes of sensors, transducers, and effectors. Many of the signaling molecules and anti-apoptotic effectors can be induced by TNF α .

8.2. TNF α pretreatment

It should no longer be surprising that the preconditioning effects of mild ischemia and hypoxia can be reproduced by TNF α pretreatment (Nawashiro *et al.*, 1997b; Liu *et al.*, 2000). TNF α pretreatment *in vitro* in an ischemia-reperfusion situation reduces the cellular expression of both TNFR1 and TNFR2 (Ding *et al.*, 2006). TNF α preconditioning produces an increase of ceramide, a protective product of sphingomyelin hydrolysis (Ginis *et al.*, 1999). This indicates that TNF α can be neuroprotective, even though it may exert a deleterious effect when working together with reactive oxygen species (Nawashiro *et al.*, 1997a; Ginis *et al.*, 2000; Hallenbeck, 2002). It is reasonable to assume, therefore, that regulation of the transport system for TNF α at the BBB may play a crucial role in the preconditioning of stroke.

The model proposed in figure 5 stresses a role of peripheral TNF α in preconditioning of the CNS to ischemic and hypoxic insults. Many cerebral vascular diseases are preceded by etiological factors affecting the peripheral organs, such as obesity, inflammation, diabetes, and hypertension. We have shown that the BBB is a communicating interface mediating the CNS effects of a variety of circulatory signals. TNF α provides an excellent tool for examination of the alteration of endothelial function that eventually affects neuronal plasticity. There are two major means of signal transmission across the BBB: direct permeation of TNF α from the microcirculation to the CNS, and generation of secondary signals that serve as diffusible factors.

9. Summary

The major theme of this review is the intricate relationships between the BBB and TNF α in the context of stroke prevention and therapeutic intervention. Although much has been shown about the CNS production and action of TNF α , the roles of the BBB in its time- and state-dependent effects certainly merit more attention. We provided evidence that circulating TNF α plays an important role in preconditioning of the neuronal response to stroke. We propose that the BBB transmits signals initiated by TNF α both by permeation through its receptor-mediated transport system and generation of secondary signals in the BBB endothelia, which can be either cytoprotective or cytotoxic.

TNF α transport involves endocytosis by both TNFR1 and TNFR2, but the mechanisms of intracellular trafficking and exocytosis require further investigation. As small amounts of TNF α can be transported across the BBB without compromising barrier integrity, delivery of TNF α or its mimetics across the BBB provides an intriguing approach for reduction of

subsequent CNS injury to a full-blown insult. This is supported by the studies showing induction of cytoprotective signals and effectors by low doses of TNF α .

The evidence reviewed illustrates that BBB disruption and tissue damage are plastic processes that undergo dynamic changes after stroke. If TNF α is beneficial at a particular time when its *in situ* production is insufficient, enhanced transport from blood to brain would facilitate the regenerative process. If excess TNF α is detrimental to stroke recovery, blockade of the key regulators of TNF α trafficking would provide a new treatment strategy. Therefore, manipulation of the transport system to achieve an optimal concentration of TNF α in the brain at appropriate times after stroke may facilitate neuroregeneration. Since TNF α is one of the key players in stroke progression, this line of research will not only identify critical modulators but also provide a template to test other proinflammatory cytokines involved in neuroregeneration.

Acknowledgements

The authors receive support from NIH (NS45751, NS46528, and DK54880).

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Abbreviations

BBB	blood-brain barrier
cIAP	cytoplasmic inhibitor of apoptosis protein
CNS	central nervous system
CSF	cerebrospinal fluid
DD	death domain
FAN	factor associated with neutral sphingomyelinase
γGT	gamma glutamyl transpeptidase
Hsp	heat shock protein
ICAM	intercellular adhesion molecule
IL	interleukin
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
tMCAO	transient middle cerebral artery occlusion
MVB	multivesicular bodies
NFκB	nuclear factor kappa B
NMDA	N-methyl-D-aspartate
NSD	neutral sphingomyelinase domain
RBE4	immortalized rat brain endothelial cell line

RIP	adaptor proteins receptor interacting protein
TNFα	tumor necrosis factor α
TNFR	TNF receptor
TRADD	TNFR-associated death domain
TRAF	TNFR associated protein
TRID	TNFR1 internalization domain

Fig.1.

Schematic presentation of signaling pathways initiated by TNFR1. There are three cytoplasmic domains: TNFR1 internalization domain (TRID) which contains a YXXW motif; neutral sphingomyelinase domain (NSD), and the death domain (DD). TNF α binding to its receptors leads to recruitment of receptor-associated proteins. The outcome may be apoptosis resulting from caspase-8 action in the mitochondria or transcriptional induction of cell survival genes. Both cytotoxic and cytoprotective actions by ceramide and NF κ B have been shown.

Fig.2.

Cellular models of receptor-mediated endocytosis of TNF α and its potential exocytosis in BBB endothelial cells. In model 1, TNFR1 and TNFR2 both mediate internalization of TNF α , probably by clathrin-mediated pathways and targeting to vesicular transport. The ligand-receptor complex dissociates at a low pH in lysosomes or multivesicular body (MVB), and the receptor undergoes recycling or degradation. TNF α is freed and may diffuse across the basolateral membrane of the cell to reach the CNS. In model 2, TNFR2 is the main mediator of TNF α trafficking resulting from its relatively higher level of expression at the cell surface. The TNF α -R2 complex moves to the TNF α -R1 complex, possibly in the Golgi complex, and is sorted to the basolateral surface.

Fig.3.

Once internalized by either receptor, TNF α undergoes two major fates: intracellular degradation or exocytosis. Even in non-polarized HEK293 cells, intact TNF α can be recovered in the exocytosis medium. TNFR2 induces more exocytosis but TNFR1 mediates a greater percent of intact TNF α .

Fig.4.

Signaling and transport of leukemia inhibitory factor (LIF) can be modulated by TNF α in cultured endothelial cells. The expression of gp190 (specific receptor for LIF) is reduced, resulting from its accelerated degradation in lysosomes. The expression of gp130 (co-receptor and signal converter for LIF) is increased, resulting from transcriptional activation of NF κ B. The overall outcome is reduced Stat3 activation and decreased LIF endocytosis.

Fig.5.

The actions of microcirculatory TNF α on the endothelial cells composing the BBB illustrate signal transmission across the BBB. The concentration of TNF α is elevated in a variety of metabolic, inflammatory, and hypoxic disorders. TNF α can be either transported across the BBB to act on the CNS directly, or it can induce secondary signals that in turn modulate CNS functions as preconditioning stimuli.