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INCREASED PERMEABILITY OF PRIMARY CULTURED BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYERS FOLLOWING TNF-α EXPOSURE

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Summary

TNF-α is a cytokine that produces increased permeability in the peripheral vasculature; however, little is known about the effects of TNF-α on the blood-brain barrier (BBB). Using primary cultured bovine brain microvessel endothelial cells (BBMEC) as an *in vitro* model of the BBB, this study shows that TNF-α produces a reversible increase in the permeability of the brain microvessel endothelial cells. The BBMEC monolayers were pre-treated with 100 ng/ml of TNF-α for periods ranging from 2 to 12 hours. Permeability was assessed using three molecular weight markers, fluorescein (376 MW), fluorescein-dextran (FDX-4400; 4400 MW), and FDX-70000 (MW 70000). The permeability of BBMEC monolayers to all three fluorescent markers was increased two-fold or greater in the TNF-α treatment group compared to control monolayers receiving no TNF-α. Significant changes in permeability were also observed with TNF-α concentrations as low as 1 ng/ml. These results suggest that TNF-α acts directly on the brain microvessel endothelial cells in a dynamic manner to produce a reversible increase in permeability. Exposure of either the lumenal or ablumenal side of BBMEC monolayers to TNF-α resulted in similar increases in permeability to small macromolecules, e.g. fluorescein. However, when a higher molecular weight marker was used (e.g. FDX-3000), there was a greater response following lumenal exposure to TNF-α. Together, these studies demonstrate a reversible and time dependent increase in brain microvessel endothelial cell permeability following exposure to TNF-α. Such results appear to be due to TNF's direct interaction with the brain microvessel endothelial cell.

Key Words: blood-brain barrier, polarity, TNF-α, reversible endothelial cell permeability, endothelial cell permeability

The brain microvessel endothelial cells that form the blood-brain barrier (BBB) have an important role in controlling the passage of solutes from the blood to the brain extracellular fluid. The

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restricted nature of the BBB is attributable to the tight extracellular junctions that limit the diffusion of molecules between the endothelial cells, and the low levels of pinocytic activity that limit the intracellular transport of molecules [1]. However, under certain conditions, the normally low permeability of the BBB can be altered, allowing macromolecules and cells of the immune system to leave the bloodstream and enter the brain extracellular fluid. Identifying the cellular factors that control BBB permeability will provide further insight into the breakdown of the BBB that occurs in immuno-inflammatory neurological diseases.

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that is released in response to bacterial and viral infections, or tissue trauma. While the biological effects of TNF-α vary depending on the cell type involved, a major target for TNF-α is the vascular endothelial cell where changes in permeability and coagulation are produced [2]. In the peripheral vasculature, release of TNF-α has been implicated in the increased vascular permeability that occurs during bacterial sepsis [3]. In the central nervous system, TNF-α has been implicated in inflammatory diseases such as meningitis, multiple sclerosis, AIDS-related dementia, stroke and brain ischemia [4-6]. Therefore, the changes in BBB permeability associated with these disease states may be attributable in part to the effects of TNF-α in the brain microvasculature.

Despite the evidence implicating TNF-α in the breakdown of the BBB, little is currently known about the microvascular endothelial cell's response to TNF-α. Early *in vivo* studies indicated that centrally administered TNF-α could cause a dose-dependent increase in BBB permeability, while systemic administration of TNF-α was less effective in disrupting the BBB [7, 8]. Separate *in vitro* studies, using brain microvessel endothelial cells co-cultured with astrocytes, demonstrated TNF-α induced increases in brain microvessel endothelial cell permeability [9]. Other *in vitro* studies show that TNF-α has no effect on brain endothelial cell permeability [10, 11]. Regardless, questions remain concerning the direct effects of TNF-α on brain microvessel endothelial cell, the potential functional polarity with regard to the permeability effects of TNF, and the reversibility of these permeability effects.

Using primary cultured bovine brain microvessel endothelial cells (BBMEC) as an *in vitro* model of the BBB, the permeability effects of TNF-α were characterized. The present studies indicate that TNF-α acts directly on the brain microvessel endothelial cell. Furthermore, the TNF-α induced increases in BBMEC monolayer permeability are completely reversible and can be produced by either lumenal (blood side) or ablumenal (brain side) exposure to the cytokine.

Methods

Isolation and Culturing of BBMEC. Primary BBMECs were collected from the gray matter of fresh cow cerebral cortices using a combination of enzymatic digestion and centrifugal separation methods previously described [12]. The BBMECs were seeded (50,000 cells/cm^2) on collagen coated, fibronectin treated, 6-well plastic tissue culture plates or 6-well Transwell® polycarbonate membrane inserts (0.4 μm pore/ 24 mm diameter). The culture media consisted of: 45% minimum essential medium eagle (MEM), 45% Ham's F12 nutrient mix, 10 mM HEPES, 13 mM sodium bicarbonate, 50 μg/ml gentamicin, 10% equine serum, 2.5 μg/ml amphotericin B, and 100 μg/ml heparin. The cell cultures were grown in a humidified 37^{0}C incubator with 5% CO_2. The media was replaced every other day, and the BBMEC monolayers were used after reaching confluency (approximately 11-14 days).

Cytokine Preparation and Treatment of BBMECs. Lyophilized recombinant human tumor necrosis factor-alpha (TNF-α; 1.1 x 10^5 U/μg; endotoxin level < 0.01%; R& D systems, Minneapolis, MN) was reconstituted to 10 μg/ml using 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and kept at -20°C until used. Working solutions of TNF-α were prepared by diluting in culture media. Prior to the permeability experiments, the BBMEC monolayers were exposed to either TNF-α (1 x 10^{-4} ng/ml - 100 ng/ml in culture media) or culture media alone. The TNF-α treated and control monolayers were kept at 37°C in a 5% CO_2 incubator for various pre-treatment periods (2, 4, 6, 8, and 12 hours). Except for the polarity studies described below, TNF-α was applied to both the lumenal and ablumenal side of the monolayers and remained present during the entire pre-treatment period.

Determination of TNF-α Effects on BBMEC Permeability. Following exposure to TNF-α, the medium was removed and the BBMEC monolayers were placed into side-by-side diffusion chambers. The monolayers were placed in the chambers so that the lumenal (apical) side faced the donor compartment and the ablumenal (basolateral) side faced the receiver compartment. Assay buffer (3 mls), consisting of MEM : Ham's F12 media supplemented with 0.1% BSA, was added to both the receiver and donor compartments. Permeability across BBMEC monolayers was determined by adding 1 μM of either fluorescein (376 MW), fluorescein-labeled dextran FDX-3000 (3000 MW), FDX-4400 (4400 MW), or FDX-70000 (70000 MW), to the donor compartment. Samples (3 mls) were removed from the receiver chamber at 0, 5, 15, 30, 60, and 120 minutes, and immediately replaced with fresh assay buffer. Samples (400 μl) from the donor chamber were taken at time zero and replaced with fresh assay buffer containing the fluorescent marker. Aliquots from the donor and receiver compartment samples were analyzed using a Shimadzu RF5000 spectrofluorometer (Ex(λ) 488 nm; Em(λ) 510 nm) to determine the amount of fluorescent marker passing across the BBMEC monolayers. The BBMEC monolayer permeability in these studies was expressed as percent transfer of the fluorescent marker, and was determined by dividing the cumulative concentration in the receiver compartment by the concentration in the donor compartment at time zero and then multiplying by 100.

Polarity Studies. In the studies examining the functional polarity of TNF-α, the cytokine was applied to the lumenal and/or ablumenal side of the BBMEC monolayers. To minimize the potential for leakage or transport of TNF-α to the opposing side of the monolayers, the TNF-α (100 ng/ml) exposure time and temperature were reduced to one hour at 4°C. After the one hour exposure, the TNF-α treated media was removed and the monolayers were incubated with fresh culture media for 6 hours at 37°C prior to the permeability studies.

Reversibility of TNF-α Induced Increases in BBMEC Permeability. To determine whether the permeability effects of TNF-α were reversible, confluent BBMEC monolayers were divided into three different groups. Each group consisted of BBMEC monolayers that were treated with TNF-α (100 ng/ml) and control monolayers that received culture media alone. All three groups were treated with TNF-α or culture media for six hours. After the six hour induction period, the media was removed from the cells and replaced with fresh culture media containing no TNF-α. The BBMEC monolayers in the first group were used in a permeability study. The remaining two groups were incubated at 37°C for 18 hours. After an 18 hour recovery period, permeability experiments were performed on the BBMEC monolayers in the second group. The BBMEC monolayers in the third group were exposed a second time to either TNF-α or culture media for six hours. Permeability experiments were conducted on these monolayers after the second TNF-α exposure.

Cytotoxicity Study. Viability of the cells following treatment with TNF-α was evaluated using the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cytotoxicity assay [13]. Confluent BBMEC monolayers were exposed to culture media or TNF-α for periods of 4, 6, 8, or 12 hours. The cells were removed using trypsin (0.05%) /EDTA (0.53 mM) and distributed evenly into a 96-well culture plate (25,000 cells/well). The cells were incubated with 5 mg/ml of MTT for two hours at 37^{0}C. The cells were solubilized in a 50:50 mixture of dimethyl formamide (DMF) and H_20 containing 20% w/v sodium dodecyl sulfate (SDS, pH 4.7). Absorbance readings were taken at 550 nm using a Microkinetics Reader™ (Fisher Biotech). Viability was expressed as a percent of control cells receiving only culture media.

Materials and Statistics. Transwell® polycarbonate membrane inserts, MEM and Ham's F-12 media were purchased from Fisher (St. Louis, MO). Bovine fibronectin, bovine serum albumin, equine serum, fluorescein, fluorescein-labeled dextran (4400 MW) were purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescein-labeled dextrans (70000 MW and 3000 MW) were purchased from Molecular Probes (Eugene, OR). Rat-tail collagen (type I) was purchased from Collaborative Biomedical Products (Medford, MA). Trypsin/EDTA was obtained from GIBCO/BRL (Grand Island, NY). All other nutrients, salts, and antibiotics used in culture media or assay buffers were of cell culture quality from Sigma.

Statistical analysis of the data from the cytotoxicity studies was evaluated using the Student's t-test. Permeability studies were analyzed by single-factor or two-factor analysis-of-variance with Newman-Keuls' multi-range post hoc comparison [14] of the means where appropriate. Permeability coefficients were calculated as described [9]. Pe is the permeability due to the endothelial cell monolayer, Pt is the permeability of the combined BBMEC monolayer and the polycarbonate filter, and Pf is the permeability of the polycarbonate membrane filter alone.

$$\frac{1}{Pe} = \frac{1}{Pt} - \frac{1}{Pf}$$

Results

Effects of TNF-α on BBMEC Monolayer Permeability. Permeability studies were performed on both BBMEC monolayers and collagen coated, fibronectin treated, polycarbonate membranes without BBMECs (blank membranes). As Figure 1 illustrates, the presence of a confluent BBMEC monolayer presents a barrier to the passage of the various fluorescent markers. Furthermore, a 6 hour pre-treatment with TNF-α (100 ng/ml), resulted in approximately a two-fold increase in BBMEC monolayer permeability to fluorescein (2.58% in control vs. 4.69% in TNF-α; Figure 1). A qualitatively similar increase in permeability was observed with the FDX-4400 and FDX-70000 markers following TNF-α exposure (FDX-4400: 0.70% in control vs. 1.35% in TNF-α; FDX-70000: 0.14% in control vs. 0.34% in TNF-α; Figure 1). The effects of TNF-α on the permeability coefficients are shown in Table 1. Permeability coefficients for the BBMEC monolayers treated with TNF-α were increased 3-6 fold depending on the marker molecule used when compared to BBMEC monolayers receiving no TNF-α.

Effects of TNF-α Exposure Time on BBMEC Permeability. The effects of TNF-α (100 ng/ml) on BBMEC permeability were examined following various exposure times as illustrated in Figure 2. There was a significant increase in BBMEC permeability at all the TNF-α exposure times examined. However, the magnitude of the permeability increase produced by TNF-α was greatest following exposures of 4 hours or more (Figure 2).

TABLE I
Permeability Coefficients of BBMEC Monolayers

	Control	TNF-α Treated
Fluorescein	1.71×10^{-3} cm/min	9.86×10^{-3} cm/min *
FDX-4400	3.70×10^{-4} cm/min	9.64×10^{-4} cm/min *
FDX-70000	1.26×10^{-4} cm/min	7.94×10^{-4} cm/min *

* Statistically significant, p-value < 0.05.

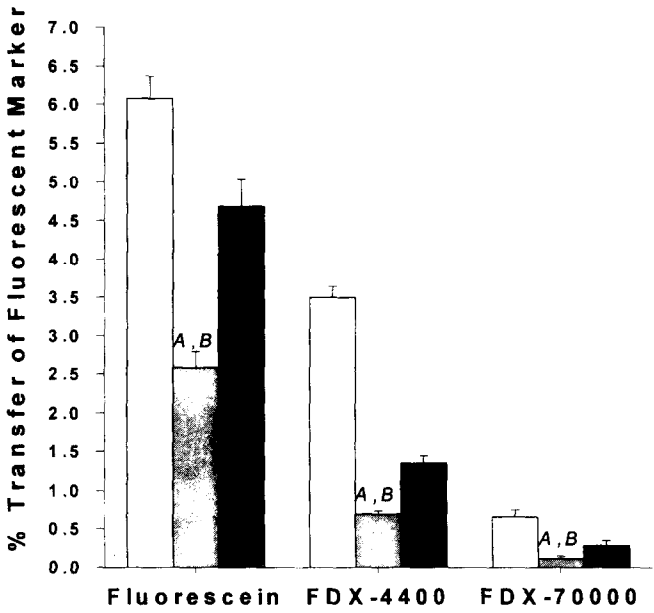

Fig. 1

Permeability of various size molecular weight markers. The permeabilities of fluorescein, FDX-4400, and FDX-70000 were examined in blank membranes (open bars), BBMEC monolayers (lt. gray bars), and TNF-α (100 ng/ml; 6 hour; dk. gray bars) treated BBMEC monolayers. Values represent the mean +/- SEM (blank: n=3, control and TNF-α treatment groups: n=6). ***A*** denotes a p-value < 0.05 compared to blank membranes, and ***B*** denotes a p-value < 0.05 compared to TNF-α treated monolayers.

Effects of TNF-α Concentration on BBMEC Permeability. To determine the minimal concentration of TNF-α required to produce an increase in BBMEC monolayer permeability, cells were exposed for 6 hours to various concentrations of TNF-α (1.0×10^{-4} ng/ml - 100 ng/ml; Figure 3). The permeability of BBMEC monolayers to fluorescein was not significantly increased following exposure to TNF-α concentrations ranging from 1.0×10^{-4} ng/ml to 0.1 ng/ml. However, significant increases in BBMEC monolayer permeability were obtained at all TNF-α concentrations greater than 0.1 ng/ml when compared to control monolayers (Figure 3).

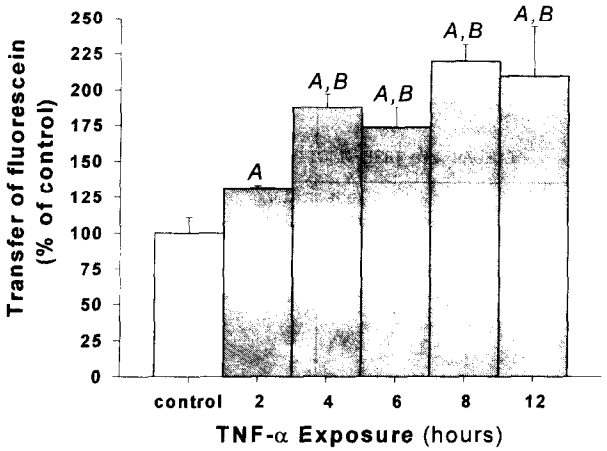

Fig. 2

Effects of TNF-α exposure time on BBMEC monolayer permeability. Confluent BBMEC monolayers were pre-treated with TNF-α (100 ng/ml) for 2, 4, 6, 8, and 12 hours (n=6; lt. gray bars) prior to permeability experiments. Results are presented as percent of control, BBMEC monolayers receiving no TNF-α (n=9; open bar). Each value represents the mean +/- SEM. ***A*** denotes a p-value < 0.05 compared to control monolayers. ***B*** denotes a p-value < 0.05 compared to 2 hour TNF-a treatment.

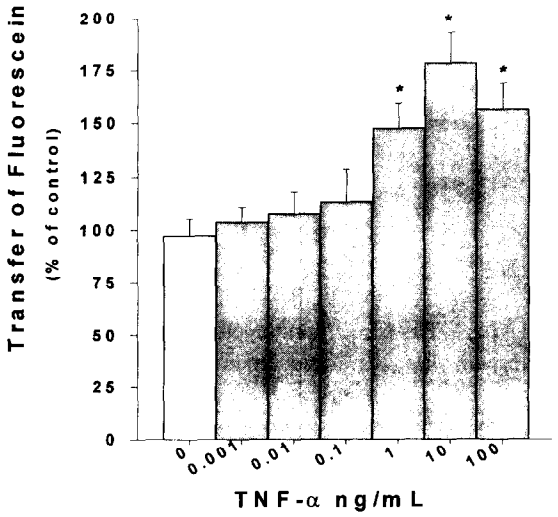

Fig. 3

Dose response of BBMEC monolayers treated with TNF-α. Confluent BBMEC monolayers were treated with various concentrations of TNF-α for six hours. Permeability studies were performed with fluorescein and the results are reported as percent transfer of fluorescein over a 2 hour period, normalized to that of control. Values represent the mean +/- SEM; n=6. TNF-α concentrations ranging from 1 -100 ng/ml were significantly different (*) with a p-value of < 0.05.

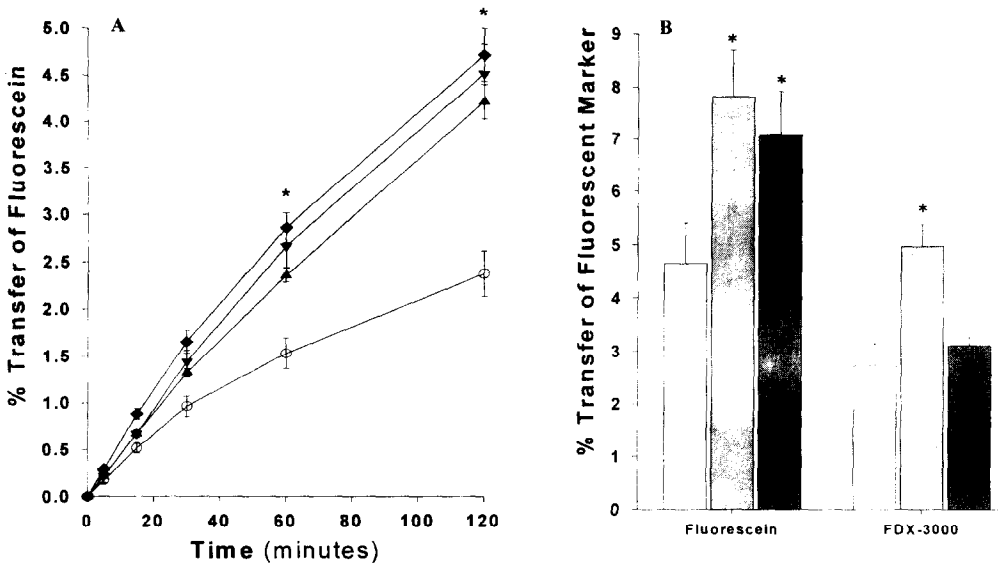

Fig. 4

Panel A: Functional polarity to the permeability effects of TNF-α. Confluent BBMEC monolayers were exposed to TNF-α (100 ng/ml, 6 hours pre-treatment) on either the lumenal (▲), ablumenal (▼), or both lumenal and ablumenal sides (◆). The flux of fluorescein across the monolayers was compared to control monolayers (O) receiving culture media alone. Values represent mean +/- SEM; n=3. Significant (p-value < 0.05) differences in permeability (*), were observed in all the TNF-α treatment groups compared to control.

Panel B: Size dependence in functional polarity to the permeability effects of TNF-α. Confluent BBMEC monolayers were exposed to TNF-α (100 ng/ml; 1 hour @ 4^0C; 6 hours @ 37^0C pre-treatment) on either the lumenal (lt. gray bars) or ablumenal (dk. gray bars) side. The flux of the fluorescent markers across the monolayers were compared to control monolayers (open bars) receiving culture media alone. Values represent mean +/- SEM; n=3. Significant (p-value < 0.05) differences in permeability are denoted (*).

Determination of Functional Polarity to TNF-α in BBMEC Monolayers. To determine if the site of TNF-α application had an affect on BBMEC permeability, TNF-α (100 ng/ml) was applied to the lumenal and/or ablumenal sides of the BBMEC monolayers. Following exposure of TNF-α to both the lumenal and ablumenal sides, fluorescein transfer increased from 2.4% in control monolayers to 4.7% in the TNF-α treated group (Figure 4A). Likewise, exposure of the monolayers to TNF-α on either the lumenal or ablumenal side produced similar increases in BBMEC monolayer permeability (4.2% and 4.5%, respectively). The effects of TNF-α on fluorescein transfer across BBMEC monolayers were observed at 15 minutes and became significant after 30 minutes (Figure 4A). In contrast, when the permeability of the BBMEC monolayers was assessed using a higher molecular weight marker, FDX-3000, a polarized response to the TNF-α became apparent. Figure 4B shows BBMEC monolayers treated with TNF-α on either lumenal or ablumenal side demonstrate a significant increase in fluorescein (376

MW) permeability compared to control (7.81% and 7.07%, respectively; vs. 4.64% in controls). In contrast, when measuring the flux of the higher molecular weight dextran (FDX-3000), the monolayers treated with TNF-α on the lumenal side demonstrated a significant increase in permeability (4.96% compared to 2.71%, control); whereas, the monolayers treated with TNF-α on the ablumenal side did not show any difference in permeability compared to control monolayers, 3.10% and 2.71% respectively.

Cytotoxicity Effects of TNF-α on BBMEC. To determine whether the increases in permeability were due to the toxic effects of TNF-α on BBMEC monolayers, cell viability was examined. Cytotoxicity studies were performed following exposure of the monolayers to 100 ng/ml TNF-α for 4, 6, 8 or 12 hours. The cytotoxicity studies showed no significant difference between control cells exposed to culture media alone, and treated cells exposed to 100 ng/ml TNF-α in culture media, at any of the exposure times examined (data not shown).

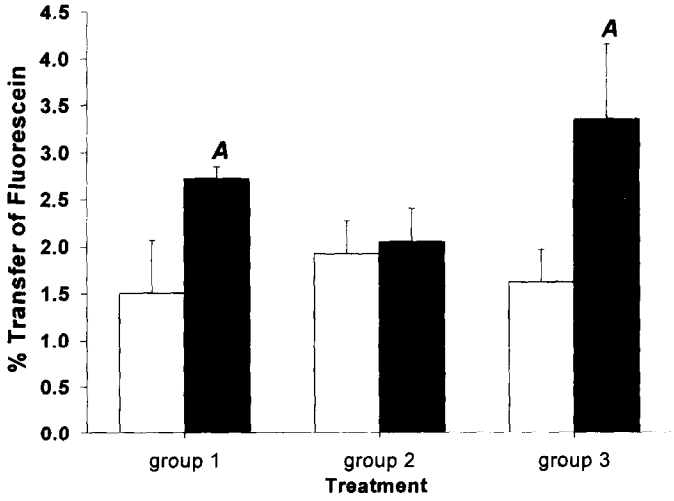

Fig. 5

Reversibility of TNF-α effects on BBMEC permeability. The permeability of BBMEC monolayers to fluorescein was examined over a 2-hour period following the various treatment protocols. Treatment group one received TNF-α (100 ng/ml; 6 hours). Treatment group two was treated with TNF-α as in group one; however, after the six hour exposure to TNF-α , the cytokine was removed and replaced with culture media for 18 hours before performing a permeability study. Treatment group three was treated the same as group two followed by a second stimulation with TNF-α (100 ng/ml; 6 hours) prior to the permeability study. Control monolayers are indicated by open bars while the TNF-α treated monolayers are indicated by the dark gray bars. Values represent the mean +/- SEM; n=3. *A* denotes a p-value < 0.05 of TNF-α treated monolayers compared to control.

Reversibility of TNF-α Induced Effects in BBMEC Monolayer Permeability. The effects of TNF-α on BBMEC permeability were examined to determine whether the response of the BBMEC monolayers to TNF-α were reversible. In these studies, 6 hour exposure to TNF-α (100 ng/ml) produced approximately a two-fold increase in BBMEC permeability (Figure 5). However, when

the TNF-α treated BBMEC monolayers (treatment group 2) were examined 18 hours after removing the cytokine, monolayer permeability had returned to control levels. Finally, exposing the BBMEC monolayers to a second treatment of TNF-α (18 hours after the first TNF-α treatment; treatment group 3) resulted in significant increases in monolayer permeability (Fig. 5).

Discussion

The effects of TNF-α on peripheral vascular permeability are well-documented [15-17]. While much of the permeability increases observed with TNF-α in the peripheral vasculature have been associated with neutrophil-dependent effects of the cytokine [18], the results of the present study indicate that TNF-α can produce a direct, reversible effect on the permeability of brain microvessel endothelial cells. This effect is both concentration- and time-dependent with optimal increases in permeability occurring with 1-100 ng/ml TNF-α at 4-12 hours post-exposure. These results suggest that the TNF-induced increases in BBB permeability reported from *in vivo* studies [7, 8, 19] are at least partially attributable to the direct effects of the cytokine on the brain microvessel endothelial cells.

It is clear from the present studies that the induction time that follows TNF-α exposure is important in determining the effects of TNF-α on BBMEC monolayer permeability. While increases in BBMEC monolayer permeability were detected 2 hours after exposure to TNF-α, maximal effects occurred 4-12 hours after exposure to the cytokine. Furthermore, 24 hours following TNF-α exposure, permeability had returned to the level observed in the control BBMEC monolayers receiving no cytokine. The four hour induction time required for the onset of the permeability effects with TNF-α in the BBMEC monolayers is similar to that reported for TNF-α in peripheral endothelial cells [15-17]. Previous studies in brain microvessel endothelial cells have been less conclusive regarding the induction period for TNF-α. Studies by deVries [20] suggest that TNF-induced increases in brain microvessel endothelial cell permeability can occur within 60-minutes. However, studies by Deli et al. [9] report no significant increases in permeability of brain microvessel endothelial cell monolayers prior to four hours following TNF-α exposure. The observation that BBMEC monolayer permeability returns to normal within 24 hours after exposure to TNF-α, helps explain why some studies report increases in brain microvessel endothelial cell permeability following TNF-α exposure [9, 20] and others, with 24 hour or longer TNF-α induction periods, do not see such increases in permeability [10].

There are two important things to note regarding the TNF-α induction time observed in the present study. First, is the lag time between TNF-α exposure and the permeability increases observed in the present study are consistent with the effects of TNF-α on BBB permeability *in vivo*. In adult rats, intracerebral injections of TNF-α produced significant increases in BBB permeability [8]. However, such increases in BBB permeability required 12-24 hours to manifest itself and were completely reversible within 72 hours. In studies using newborn pigs, Abraham and colleagues [19] showed significant increases in BBB permeability to Evan's blue-albumin with 2 hours exposure to TNF-α. Of possibly equal importance is the observation that those *in vivo* studies which report no change in BBB permeability following TNF-α administration [21, 22] have relatively short induction periods, i.e., less than 2 hours.

The second point regarding the TNF-α induction period observed in the current study is that exposure to TNF-α is not required throughout the entire induction time. This is evident from the increases in permeability observed in the polarity studies where BBMEC monolayers were exposed to TNF-α for only the first hour of the 6 hour induction period. This suggests that the

permeability effects observed are not the product of additive effects due to prolonged exposure to the cytokine.

Significant increases in BBMEC monolayer permeability were observed in the present study with concentrations of TNF-α ranging from 1-100 ng/ml. This is in agreement with previous studies examining the effects of TNF-α on peripheral [15, 16] and brain endothelial cell permeability [9, 20] The pathophysiological relevance of the concentrations of TNF-α required to produce a change in BBMEC monolayer permeability is unclear. However, clinical studies have reported TNF-α levels in the 0.1-1.0 ng/ml range in the serum and cerebrospinal fluid of patients suffering from neurological conditions such as multiple sclerosis [5], Alzheimer's disease [23], and AIDS related dementia [6]. Due to a diluting effect from the CSF and plasma, the amount of TNF-α at the localized site of inflammation or injury may actually be greater than the concentration measured. Taking this possibility into account, the concentrations of TNF-α used in this study would appear to be comparable to levels of TNF-α that could be expected to occur within the vasculature during various pathophysiological conditions in the CNS [5, 6, 23]. Therefore, the effects of TNF-α on BBMEC monolayer permeability observed in the present study may be indicative of the effects produced by the cytokine on BBB permeability under pathological conditions elicited by inflammatory responses.

The nature of the permeability effects observed with TNF-α would appear to support an increase in diffusion between the endothelial cells, i.e., enhanced paracellular diffusion. Evidence supporting this is the observation that the effects of TNF-α in the polarity studies were dependent on the size of the permeability marker used. If increases in permeability following TNF-α exposure were occurring through intracellular transport routes as has been demonstrated previously [21], one would have anticipated that the ablumenally applied TNF-α to have similar permeability effects for both the fluorescein and dextran markers. This was not observed in this study. For such an increase in paracellular diffusion to occur, it would require the breakdown of the tight junctions that exist between the cells. Alterations in cytoskeletal structure have been reported previously in endothelial cells exposed to TNF-α [9]. In addition, actin filament staining in the present study appears to support the formation of actin stress fibers tangles and extracellular gaps in the TNF-α treated BBMEC monolayers (data not shown).

While the formation of the extracellular gaps appear to be due to re-arrangement of the cytoskeleton, the cellular events that control this event remain unknown. One possibility is that TNF-α is activating the arachadonic acid metabolism pathway resulting in the formation of prostaglandins and/or leukotrienes. Previous studies have suggested possible activation of the arachadonic acid pathway in describing the effects of TNF-α [20]. Furthermore, there are some indications that the arachadonic acid pathway can result in alterations in cytoskeletal structure [24]. The involvement of this cellular signaling pathway in the TNF-mediated increases in BBMEC monolayer permeability are currently under investigation. An alternative cellular signaling pathway involved in the TNF-induced permeability effects may be the production of nitric oxide [25]. In this regard, studies by Bonmann et al. report an increase in the expression of inducible nitric oxide synthase in rat brain microvessel endothelial cells following exposure to combinations of TNF-α and other cytokines. Regardless of the exact mechanism, the latency period required for the TNF-mediated increases in permeability in the BBMEC monolayers suggests that a cascade of signaling events are likely to occur and that TNF-α interactions with the BBMEC is an upstream event in this process.

While the cellular mechanism(s) involved in the permeability response to TNF-α remain unknown, the results of the present study indicate that the effects observed are not due to cytotoxicity to the TNF-α. Evidence in support of this is two-fold. First, cell viability studies show no toxicity to 100 ng/ml TNF-α in the BBMEC. Secondly, the permeability increases observed with TNF-α in the present study are fully reversible within 24 hours after exposure to the cytokine. Together, these results suggest that the permeability increases observed with TNF-α is a dynamic and reversible response to the cytokine.

The brain microvessel endothelial cells that form the BBB display asymmetry, i.e., polarity in the plasma membrane distribution of many transporters and receptors [1]. While there appears to be a polarized lumenal to ablumenal transport system for TNF-α in the BBB [21], the current study is the first to examine potential functional polarity to the permeability effects of TNF-α in BBMEC monolayers. The data indicate that for small molecular weight compounds such as fluorescein, there is no polarity in response to TNF-α. However, when a larger molecular weight molecule is used, there is a clear polarized response to TNF-α, with only lumenal exposures producing a significant enhancement of BBMEC monolayer permeability. Based on these studies it would appear that there is an increased response to lumenal application of TNF-α compared to ablumenal exposure. However, the polarity observed appears to be subtle requiring larger molecular weight permeability markers to become apparent.

The results from the polarity studies are dependent upon minimizing the leakage of TNF-α across the BBMEC monolayer. It should be noted that similar techniques have been used previously to demonstrate the polarized distribution of insulin receptors in the BBMEC [26]. For the present study, the size and physico-chemical properties of TNF-α would appear to preclude the cytokine from passively diffusing across the BBMEC monolayers to any appreciable extent. However, to ensure minimal leakage for the polarity studies, BBMEC monolayers were exposed to TNF-α for only 1 hour at 4^0C. By incubating the cells with TNF-α at 4^0C, potential receptor-mediated transport of TNF-α, as has been reported previously in the BBB [21], was also inhibited. Therefore, it would appear that at the level of the brain endothelial cell, lumenal exposure to TNF-α may produce a greater response than ablumenal exposure.

Identification of potential polarity issues with regard to TNF-induced increases in BBMEC monolayer permeability has clinical ramifications. Tumor necrosis factor is found in a variety of cells including macrophages, T and B lymphocytes, fibroblasts, astrocytes, glial cells, and endothelial cells [27]. Therefore, it is possible that the TNF-α required to increase BBB permeability could be released from cells within the central nervous system, i.e., astrocytes and glial cells, or from cells in the periphery, i.e., leukocytes and fibroblasts, or from brain endothelial cells themselves. Increases in TNF-α concentrations are found in central (CSF) and/or peripheral (blood) compartments during such pathophysiological conditions as multiple sclerosis [5], Alzheimer's [23], and AIDS related dementia [6]. So the question becomes, is the BBB more sensitive to centrally or peripherally released TNF-α? The results from *in vivo* studies is rather inconclusive as increases in BBB permeability have been reported following either peripheral (intracarotid) [8] or central administration [7] of TNF-α. Based on the present studies, it would appear that the brain microvessel endothelial cells could respond to either peripheral or centrally released TNF-α, although the magnitude of the response may be greater from plasma TNF-α compared to TNF-α in the CSF.

In summary, the present studies demonstrate that TNF-α can produce permeability increases in BBMEC monolayers. The permeability increases observed with TNF-α are fully reversible and

dependent on both the concentration of the cytokine and time following exposure. While TNF-α can elicit permeability responses when applied to either the lumenal or ablumenal side of the BBMEC monolayers, there appears to be a greater responsiveness to lumenal exposure of TNF-α. Understanding the cellular events that lead to the cytoskeletal and permeability changes observed with TNF-α in the BBMEC monolayers should provide useful information for the treatment and prevention of inflammatory diseases involving the central nervous system.

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