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Role of Caspases in Cytokine-Induced Barrier Breakdown in Human Brain Endothelial Cells

M. Alejandro Lopez-Ramirez,* Roman Fischer,† Claudia C. Torres-Badillo,* Heather A. Davies,* Karen Logan,* Klaus Pfizenmaier,† David K. Male,* Basil Sharrack,‡ and Ignacio A. Romero*

During neuroinflammation, cytokines such as TNF-α and IFN-γ secreted by activated leukocytes and/or CNS resident cells have been shown to alter the phenotype and function of brain endothelial cells (BECs) leading to blood–brain barrier breakdown. In this study, we show that the human BEC line hCMEC/D3 expresses the receptors for TNF-α, TNF receptor 1 and TNF receptor 2, and for IFN-γ. BEC activation with TNF-α alone or in combination with IFN-γ induced endothelial leakage of paracellular tracers. At high cytokine concentrations (10 and 100 ng/ml), this effect was associated with caspase-3/7 activation and apoptotic cell death as evidenced by annexin V staining and DNA fragmentation (TUNEL) assays. In addition, inhibition of JNK and protein kinase C activation at these doses partially prevented activation of caspase-3/7, although only JNK inhibition was partially able to prevent the increase in BEC paracellular permeability induced by cytokines. By contrast, lower cytokine concentrations (1 ng/ml) also led to effector caspase activation, increased paracellular flux, and redistribution of zonula occludens-1 and VE-cadherin but failed to induce apoptosis. Under these conditions, specific caspase-3 and caspase-9, but not caspase-8, inhibitors partially blocked cytokine-induced disruption of tight and adherens junctions and BEC paracellular permeability. Our results suggest that the concentration of cytokines in the CNS endothelial microenvironment determines the extent of caspase-mediated barrier permeability changes, which may be generalized as a result of apoptosis or more subtle as a result of alterations in the organization of junctional complex molecules. *The Journal of Immunology, 2012, 189: 3130–3139.

Blood–brain barrier (BBB) dysfunction is a hallmark of neuroinflammatory diseases such as HIV encephalitis, bacterial meningitis, and multiple sclerosis (MS) (1–3). Areas of inflammation in MS brains show increased brain endothelial leakage, which might precede cerebral endothelial dysfunction allowing activated leukocytes and inflammatory mediators to enter the CNS (1, 4), contributing to the onset, progression of, and/or worsening of relapses (5).

Brain endothelial cells (BECs) are the first physical barrier to the CNS and express junctional complexes, including tight junctions (TJs) and adherens junctions (AJs) (6). TJ components such as claudins (1, 3, 5, and 12), occludin, and junctional adhesion molecules interact with the actin cytoskeleton by scaffolding proteins such as zonula occludens-1 (ZO-1) forming a continuous belt-like structure that can be discerned by immunofluorescence. The continuity of TJs and the sealing of the paracellular cleft confer BECs with two BBB features, namely the “gate function,” which restricts paracellular permeability, and the “fence function,” which limits the free diffusion of lipids and anchor proteins between the apical and basolateral membrane (7, 8). Additionally, AJ proteins, including VE-cadherin, mediate cell to cell contact that supports the barrier properties and regulates TJ expression in BECs (9, 10).

In active inflammatory lesions in MS, BECs show focal abnormalities in the distribution of occludin and ZO-1 including absence or punctuated immunostaining on junctions and increased localization in the cytoplasm (4, 11). Decreased expression and focal degradation of VE-cadherin has been also reported to occur in active MS lesions (9). Furthermore, during neuroinflammation, infiltrated leukocytes and brain resident cells create localized microenvironments with high levels of inflammatory mediators such as TNF-α and IFN-γ (12). TNF receptor 1 (TNFR1) is expressed constitutively on almost every nucleated cell type and tissue, whereas the expression of TNF receptor 2 (TNFR2) is highly regulated with prominent expression in cells of the immune system and endothelium, including BECs (13, 14). Increased levels of TNF-α have been reported not only to modulate endothelial TJs and cytoskeleton rearrangement in BECs (15, 16) but also to evoke an increase in caspase-3 activation driving BECs into apoptosis (13, 17). More recently, a study has proposed that primary cultures of human BECs are resistant to cell death mediated by TNF-α (18). However, caspase activation does not always lead to cell death (19), and it has been shown that hypoxia can induce caspase-9 and caspase-3 activation without apoptosis in neurons (20). Additionally, caspase-3 has been shown to be involved in ZO-1 and claudin-5 disassembly from the intercellular junctions independently of nuclear fragmentation during cerebral ischemia (21).

In the current study, we describe the subcellular localization of cytokine receptors TNFR1, TNFR2, and INFGR on the human
cerebral microvascular endothelial cell line, hCMEC/D3, and show that TNF-α alone or in combination with IFN-γ induces hyperpermeability to paracellular tracers. The BEC barrier breakdown at high concentrations of cytokines correlates with caspase-3/7 activation via JNK and protein kinase C (PKC) signaling pathways and with an increase in the number of cells undergoing apoptosis. We also demonstrate that at low concentrations of cytokines, caspase-9 and caspase-3 inhibitors partially prevent both disruption of junctional complexes and increased paracellular permeability in the absence of apoptosis. We propose that cytokines have a dose-dependent effect on brain endothelial barrier function in CNS inflammation. BECs exposed to high concentrations of cytokines might be susceptible to caspase-mediated apoptosis, whereas lower concentrations of cytokines that do not result in apoptosis promote barrier breakdown by inducing alterations in the organization of junctional complexes.

Materials and Methods

Materials

TNF-α and IFN-γ were obtained from R&D Systems (Abingdon, Oxon, U.K.). Rabbit polyclonal anti-TNFFR1 (HP9002) and anti-TNFFR2 (HP887) Abs were obtained from Hycult Biotech (Uden, The Netherlands). Mouse anti-IFNFR1 (CD119; MCA1450T) Ab was obtained from AbD Serotec (Kidlington, U.K.), and anti–VE-cadherin (D8772) rabbit polyclonal Abs were obtained from Cell Signaling (New England Biolabs, Hitchin, Hertfordshire, U.K.). Polyclonal anti–ZO-1 (61-7300), anti-occludin (40-7400), and anti–claudin-5 (34-1600) Abs were obtained from Zymed Laboratories (Sarasota, FL). Goat anti–ZO-1 (43-1000), anti–VE-cadherin (43-2000), anti-occludin (43-2100), and anti–claudin-5 (43-2400) Abs were obtained from BD Biosciences (Oxford, U.K.). All other reagents were purchased from Sigma-Aldrich (Gillingham, Dorset, U.K.), unless otherwise specified.

Cell culture

hCMEC/D3 cells at passages 23–33 were routinely cultured in EGM-2 MV medium (Lonza, Slough Wokingham, U.K.); hereafter referred to as EGM-2 medium and supplemented with supplements obtained from the manufacturer at the following concentrations: 0.025% (v/v) rhEGF, 0.025% (v/v) VEGF, 0.025% (v/v) IGF, 0.1% (v/v) rhFGF, 0.1% (v/v) gentamicin, 0.1% (v/v) ascorbic acid, 0.04% (v/v) hydrocortisone, and 2.5% (v/v) FBS as specified by Weksler et al. (22). Tissue culture flasks were precoated with 1/100 collagen type I solution. Cells were then seeded onto collagen-coated flasks and maintained at 37°C in 5% air and 5% CO2 until confluence.

Immunofluorescence

hCMEC/D3 cells were grown to confluence on collagen-coated Lab-Tek chamber slides, washed twice with prewarmed HBSS, and fixed for 10 min at room temperature with 4% paraformaldehyde in PBS pH 7.4. Slides were blocked with 0.5% BSA for 30 min and incubated with anti–TNFR1 (1:100), anti–TNFR2 (1:100), or anti-IFNFR1 (1:200) Abs for 1 h at room temperature. Cells were washed with PBS and incubated for 1 h at room temperature with a secondary goat anti-rabbit or anti-mouse Alexa Fluor 488 or 546 Ab (Zymed, Life Technologies Invitrogen division, Paisley, U.K.). To determine the expression of junctional complex molecules, cells were either unstained or stimulated with the indicated cytokines in EGM-2 media without VEGF, washed as described earlier, fixed, blocked with BSA, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Cells were then incubated with anti–ZO-1 (1:80) or anti–VE-cadherin (1:300) Abs at room temperature overnight in a humidified box followed by incubations with appropriate fluorescence-labeled secondary Abs. Cell nuclei were stained with DAPI using mounting media obtained from SouthernBiotech (Birmingham, AL). Slides were observed in a fluorescent microscope (Olympus BX61; Olympus, Hertfordshire, U.K.) or confocal microscope (Leica Microsystems, Mannheim, Germany) and images captured using Cell^P or Leica application suite software, respectively.

Flow cytometry

Confluent hCMEC/D3 cells were washed in HBSS without Ca2+/Mg2+ and harvested using 0.25% trypsin–EDTA. For cytokine receptor expression, cells were fixed and stained as earlier for TNFR1, TNFR2, and IFNFR1. To identify early apoptotic events, cells were treated with the indicated cytokine concentrations, washed, trypsinized, and stained with an annexin V–FITC apoptosis detection kit (Calbiochem Merck Biosciences, Nottingham, U.K.) following the manufacturer’s instructions. Cells were finally suspended in 500 μl PBS, and 10,000 cells were counted per experiment using a Becton Dickinson FACScan, and the data were analyzed using Cell Quest software.

Paracellular permeability and transendothelial electrical resistance

Cells were seeded onto collagen- and fibronectin-coated permeable poly-ester Transwell filter inserts (0.4-μm pore, 12-mm diameter; Corning Costar, Buckingham, U.K.) and maintained for 72 h after confluence. Culture media was then changed to EGM-2 media without VEGF, and TAT-TI-JIP153-163 and bisindolylmaleimide-1 (Bio-1) inhibitors (Calbiochem Merck Biosciences, Nottingham, U.K.) were added 40 min prior to cytokine treatment. Cells were then treated with TNF-α alone or in combination with IFN-γ at the concentrations and times indicated for each experiment. Culture media was then removed from the apical chamber, and 500 μl of transport buffer (2% FBS in DMEM without phenol red) containing 2 mg/ml 70 kDa FITC-dextran was added. Paracellular flux of tracer to the lower chamber containing 1.5 ml of transport buffer was measured sequentially at 5-min intervals for 30 min at λexc 485 nm and λem 525 nm on a BMG plate reader (Ortenberg, Germany). The volume cleared was plotted against time, and the slopes of the curves were used to calculate the permeability coefficients (Pe, cm/min) of the endothelial monolayers as previously described (23, 24). The transendothelial electrical resistance (TEER) of hCMEC/D3 monolayers was determined using an Endohm 12 chamber and an Endohmeter (World Precision Instruments, Sarasota, FL).

Western blot assays

For Western blotting of junctional complex molecules, hCMEC/D3 cells were grown to confluence in 6-well plates. Cells were lysed by scraping into a lysis solution containing 400 μl RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) and a mixture of inhibitors (5 μg/ml of aprotinin, leupeptin, pepstatin and 1 mM sodium orthovanadate). After sonication, the amount of protein was determined using a Bio-Rad, Hermit CD protein assay (Bio-Rad, Hercules, CA). Lysates were then diluted to 1× Laemmli’s buffer solution at 95°C for 10 min. Twenty-five micrograms of total protein was run per lane on 10% or 12% SDS-PAGE gels and transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, U.K.) using a wet transfer system (Bio-Rad, Hemel Hempstead, U.K.). Monoclonal antibodies were blocked with 5% non-fat milk/0.5% Tween-20 in PBS for 1 h and incubated in the presence of anti–ZO-1 (1:150), anti–VE-cadherin (1:500), anti-occludin (1:170), or anti–claudin-5 (1:170) Abs at 4°C overnight and then with a species-specific secondary anti-rabbit IgG (1:3000; Zymed) or mouse IgG (1:14000; Pierce Biotechnology, Cheshire, U.K.) Ab conjugated to HRP for 1 h at room temperature. Immunoblots were then developed by ECL detection (Amersham, Buckinghamshire, U.K.). As a control for protein loading and transfer, membranes were stripped in 2% (w/v) SDS, 0.0625 M Tris pH 6.8, 0.008% (v/v) 2-mercaptoethanol for 40 min at 50°C and incubated with mAb against actin (1:200) (A1978, Sigma-Aldrich, Gillingham, Dorset, U.K.) or anti-GAPDH (1:3000) (AM4300; Applied Biosystems, Foster City, CA) at 25°C for 10 min. Bands were visualized and quantified using ImageJ software.

Reverse transcription quantitative PCR analysis

hCMEC/D3 cells were washed once with prewarmed HBSS. Total RNA was then extracted using TRizol reagent (Invitrogen, Paisley, U.K.) according to the manufacturer's protocol. To determine the concentration and purity of RNA, 2 μl of each sample was dissolved in 500 μl 10 mM Tris-HCl solution pH 7.5, and samples were analyzed by UV spectrophotometry at 260 and 280 nm using a GeneQuantuo spectrophotometer (Amersham Biosciences, Buckinghamshire, U.K.). cDNA was obtained using reverse transcriptase (Promega, Madison, WI) with random primers according to the manufacturer's protocol. Briefly, 1 μg RNA was added to a master mix containing 5 mM MgCl2, 1× reverse transcription buffer, 1 mM each deoxynucleotide triphosphates, 1 U/μl recombinant RNase inhibitor, 15 U/μg AMV reverse transcriptase, and 0.5 μg random primers. The mixture was then placed in a thermal cycler (Bio-Rad, Hercules, CA) at 25°C for 10 min and then incubated at 42°C for 15 min. To incorporate the AMV reverse transcriptase, samples were heated at 95°C for 5 min and maintained at 4°C or −20°C. SYBR Green real-time PCR (SABiosciences, Frederick, MD) was used to determine the relative levels of the genes analyzed. Specific primers provided by SABiosciences were...
used to determine human claudin-5 (PPH22771A) and occludin (PPH02571A) mRNA levels, whereas actin (PPH00073A) mRNA levels were used as an internal control. The reaction was then placed in a thermal cycler (DNA engine Opticon 2; Bio-Rad, Hercules, CA) using an initial step at 95°C for 15 min, followed by 40 cycles (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C). The 2^{-\Delta\Delta C_T} method was used for analysis of the data (25). Each control value was normalized to one, and treatment values were relative to control.

Caspase activity assay

Detection of caspase-3/7 activity in hCMEC/D3 cells was performed using ApoTox-Glo Triplex Assay according to the manufacturer’s protocol (Promega, Madison, WI). Cells were grown on collagen-coated white 96-well cell culture microplates (Greiner Bio-One). Luciferase activity was detected using a BMG plate reader (Ortenberg, Germany).

TUNEL assay

hCMEC/D3 cells were seeded onto collagen- and fibronectin-coated coverslips and maintained for 96 h after confluence. Cells were then treated with TNF-α alone or in combination with IFN-γ (Immunotools, Friesoythe, Germany) at the concentrations and times indicated. After 24 h, cells were washed and fixed with 0.1% Triton X-100 in PBS for 5 min. Labeling with the TUNEL reaction mix was performed following the manufacturer’s protocol (Roche Applied Science, Mannheim, Germany). Coverslips were mounted with Fluoromount G (SouthernBiotech, Birmingham, AL), and the fluorescence was analyzed by fluorescence microscopy (CellObserver; Carl Zeiss).

Nuclear protein purification

Nuclear protein extracts were obtained from hCMEC/D3 cells according to the method of Staal et al. (26). Briefly, the cells were grown to confluence for 48 h and stimulated with TNF-α alone or in combination with IFN-γ for 30 min. Cells were washed twice in ice-cold PBS and scraped into 0.4 ml cell lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl_2, 1 mM DTT, 0.1 mM EDTA, 0.4 mM PMSE, 0.2 mM NaF, 0.2 mM Na orthovanadate, and protease inhibitors). The cells were allowed to swell on ice for 15 min before 25 μl 10% Nonidet P-40 was added, and the cells were vortexed vigorously for 15 s and centrifuged for 30 s in a microcentrifuge (14,000 rpm). The nuclear pellet was resuspended in 50 μl nuclear extraction buffer (50 mM HEPES, pH 7.8, 50 mM NaCl, 300 mM NaF, 0.1 mM EDTA, 1 mM PMSF, 10% [v/v] glycerol, 0.2 mM NaF, and 0.2 mM Na orthovanadate) by pipetting up and down or rotating on a platform (4°C) for 20 min. The samples were centrifuged for 5 min at 14,000 rpm and the supernatants removed and stored at −80°C. The protein concentrations were determined using the Bio-Rad protein assay following the manufacturer’s protocol.

EMSAs

Small double-stranded oligonucleotides containing AP-1 (cat. no. E320B) or NF-κB (cat. no. E329B) consensus sites (Promega, Madison, WI) were end-labeled with [γ-32P]ATP (Amersham, Buckinghamshire, U.K.) using T4 polynucleotide kinase at 37°C for 30 min. The labeled double-stranded oligonucleotides were purified through a Probe Quant G50 microcolumn (Promega, Madison, WI) into a final volume of 30 μl. Five micrograms of nuclear extracts were incubated in 20 μl DNA binding buffer, containing 20 mM HEPES (pH 7.5), 4% Ficoll, 1 μg poly (dl-dC),

FIGURE 1. hCMEC/D3 cells express the receptors for proinflammatory cytokines. (A) The level of expression of TNF-α and IFN-γ receptors in unstimulated cells (open histogram) was determined by flow cytometry in comparison with a negative control Ab (filled histogram). (B) Immunofluorescence microscopy was used to determine the presence of TNF-α and IFN-γ receptors in unstimulated, confluent hCMEC/D3 cells (left). Right panels show nuclear staining with DAPI in the same field (n = 2). Scale bar, 20 μm. Data are from one representative experiment.
0.1 mM MgCl₂, 0.1 mM DTT, and 1 μl ³²P-end-labeled double-stranded oligonucleotide probe (~10,000 dpm or 10 fmol) for 35 min at room temperature. After mixing with 1 μl loading buffer (250 mM Tris-HCl, pH 7.8, 0.2% bromophenol blue, 40% glycerol), the resulting DNA–protein complexes were separated from free oligonucleotide by subjecting them to electrophoresis in a precooled and pre-run 4%, nondenaturing polyacrylamide gel (29:1, acrylamide/bisacrylamide) and electrophoresed in 0.25× TBE buffer for 10 min at 240 V and a further 5 h at 120 V. After electrophoresis, the gel was dried under vacuum for 45 min at 80˚C and visualized by autoradiography by exposure to Kodak X-Omat film at −70˚C with intensifying screens.

**Statistical analysis**

Data are represented as means ± SEM. The number of independent experiments, n, is indicated. Statistical significance was considered if p was <0.05 determined by Student t test, two-tailed and paired.

**Results**

**Expression of TNF-α and IFN-γ receptors by hCMEC/D3 cells**

To analyze the effects of TNF-α and IFN-γ on BECs, we first identified whether the human cerebral microvascular endothelial cell line hCMEC/D3 expressed the appropriate cytokine receptors on the cell surface. TNFR1 and TNFR2 as well as IFNGR1 were expressed at the cell surface, as indicated by flow cytometry (Fig. 1A).

To determine the subcellular distribution of cytokine receptors, hCMEC/D3 cells were fixed, and the expression of non-permeabilized TNF-α receptors on the plasma membrane was analyzed by immunocytochemistry using fluorescent microscopy (Fig. 1B). Under basal conditions, TNFR1 (p55) and TNFR2 (p75) were distributed uniformly throughout the cell surface of hCMEC/D3 cells (Fig. 1B). By contrast, IFNGR staining appeared as a punctate pattern on the plasma membrane of the hCMEC/D3 cells, suggesting that IFNGR is located in clusters (Fig. 1B). These results are consistent with previous reports showing that IFNGR on HeLaM cells is compartmentalized in lipid-enriched microdomains (27). These results indicate that hCMEC/D3 cells are a suitable BBB in vitro model to study TNF-α– and IFN-γ–mediated activation of signaling pathways and their effect on paracellular permeability.

**TNF-α and IFN-γ modulate paracellular permeability and transendothelial electrical resistance of hCMEC/D3 cells**

Previous studies have shown that treatment of human peripheral microvascular endothelial cells with either TNF-α alone or in combination with IFN-γ induces an increase in paracellular permeability (28–30). To evaluate the capacity of proinflammatory cytokines to disrupt the gate properties in hCMEC/D3 cells, we performed paracellular permeability studies using the hydrophilic tracer 70 kDa FITC–dextran. After treatment with 10 ng/ml TNF-α, the permeability coefficient (Pe) in hCMEC/D3 monolayers grown on filters was increased 5-fold at 24 h of treatment compared with control cells (Fig. 2A), and this effect was further increased with combination of TNF-α and IFN-γ (10 ng/ml of each cytokine) (Fig. 2A). In addition, treatment with TNF-α and IFN-γ induced an earlier increase in the paracellular permeability of hCMEC/D3 cells detectable 6 h after treatment (Fig. 2A). By contrast, no change in hCMEC/D3 paracellular permeability was observed after 2 h of challenge with either treatment compared with control (Fig. 2A).

Increasing the cytokine concentration above 10 ng/ml did not induce any further paracellular leakage at 24 h (Fig. 2B). Conversely, paracellular permeability induced by 1 ng/ml TNF-α alone or in combination with IFN-γ for 24 h was increased to a lesser extent than that observed with 10 ng/ml cytokines (Fig. 2B). These data indicate that both low and high concentrations of TNF-α either singly or in combination with IFN-γ may induce breakdown of the hCMEC/D3 cell barrier leading to leakage of the paracellular tracers.

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Data are represented as means ± SEM. The number of independent experiments, n, is indicated. Statistical significance was considered if p was <0.05 determined by Student t test, two-tailed and paired.

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In addition, the TEER was determined in hCMEC/D3 monolayers. Baseline TEER values were ∼10 Ω cm² in our culture conditions, slightly lower than those previously reported (∼40 Ω cm²) (22). Furthermore, after treatment with TNF-α alone or in combination with IFN-γ for 24 h, the TEER of hCMEC/D3 monolayers was decreased ∼1.4- to 1.8-fold compared with control cells at all concentrations tested (Fig. 2C). These data indicate that even though cytokines induce small significant TEER reductions in hCMEC/D3 cells, this assay is not sensitive enough to study molecular mechanisms underlying barrier breakdown in this cell line.

Changes in paracellular permeability and TEER correlated with changes in the expression of tight junctional proteins at the transcript and protein level. TNF-α alone or in combination with IFN-γ induced downregulation of occludin and claudin-5 mRNA levels in a dose- and time-dependent manner (Supplemental Figs. 1A, 1B, 2C, 3C). Decreased claudin-5 protein levels were detected after 6 h of treatment with TNF-α alone (10 ng/ml), and low levels were maintained until the end of the 24-h experimental period (Supplemental Fig. 3A, 3B). By contrast, treatment of hCMEC/D3 cells with TNF-α and IFN-γ evoked downregulation of both occludin and claudin-5 mRNA and protein levels (Supplemental Figs. 2, 3).

**JNK signaling inhibitors partially prevent cytokine-induced increase in paracellular permeability in hCMEC/D3 cells**

To investigate the molecular mechanisms that contribute to the cytokine-induced increase in paracellular permeability, we used inhibitors of the canonical pathways of TNF-α signaling. TNF-α alone or in combination with IFN-γ induced activation of NF-κB and JNK signaling pathways in hCMEC/D3 cells (Supplemental Fig. 4A, 4B). Inhibitors of NF-κB (50 μg/ml SN50, IkB kinase inhibitor; Merck) had no effect on cytokine-induced hyperpermeability in hCMEC/D3 cells after 24 h of stimuli (data not shown). It is known that JNK, a member of the MAPKs, is activated after stimulus with TNF-α (31, 32). To determine the role of JNK activation, we used the JNK inhibitors SP600125 (50 μM), which effectively inhibits the phosphorylation of c-Jun (33), and the permeable peptide TAT-TI-JIP153–163 (10 μM), a competitive inhibitor of c-Jun for JNK (34). Pretreatment with SP600125 or TAT-TI-JIP153–163 partially prevented TNF-α-induced endothelial barrier dysfunction after 24 h of stimulus (Fig. 3A). Similar results were observed when hCMEC/D3 cells were pretreated with JNK inhibitors after stimulation with a combination of TNF-α and IFN-γ for 24 h (Fig. 3A). Furthermore, TNF-α-mediated JNK activation has been shown to depend on PKC signaling in endothelial cells (35). However, using a pan-PKC inhibitor Bis-1 (5 μM), we only observed a tendency to reverse the increased paracellular permeability induced by TNF-α and IFN-γ (Fig. 3B). These results suggest that JNK signaling contributes to cytokine-induced hyperpermeability in hCMEC/D3 cells after long-term stimulation with cytokines.

**Proinflammatory cytokines induce caspase-3/7 activation in hCMEC/D3 cells**

Previously, it has been shown that sustained JNK activation induces endothelial cell death by apoptosis (36), and apoptotic cell death has been proposed as a mechanism mediating increased brain endothelial permeability (37). However, it has been reported that primary cultures of human BECs are resistant to cell death mediated by TNF-α (18). To investigate whether cytokine-induced increased permeability in hCMEC/D3 cells was mediated at least partially by apoptotic cell death, we determined the effect of TNF-α alone or in combination with IFN-γ on effector caspase activation, annexin V binding, and DNA fragmentation (TUNEL) assays. Treatment with TNF-α at 10 and 100 ng/ml for 24 h induced a 2-fold increase in caspase-3/7 activity and a 1.4-fold increase in annexin V binding, whereas only TNF-α concentrations...
of 100 ng/ml induced an ∼2.7-fold increase in the percentage of TUNEL+ cells compared with unstimulated hCMEC/D3 cells (Fig. 4). The TNF-α–induced apoptotic response, in particular that of TUNEL+ cells, was further increased in combination with IFN-γ (activated caspase-3/7, ∼2.5-fold; annexin V+ cells, ∼1.8-fold; TUNEL+ cells, ∼5- and ∼12-fold at 10 ng/ml or 100 ng/ml TNF-α and IFN-γ) (Fig. 4). Notably, low concentrations of TNF-α alone (1 ng/ml) or in combination with IFN-γ (1 ng/ml) for 24 h also induced increases in effector caspase-3/7 activity (1.4- and 1.7-fold, respectively, over control), but did not increase the number of apoptotic cells, as revealed by annexin V binding or TUNEL staining.

We then determined whether the apoptotic effect of TNF-α alone or in combination with INF-γ at different concentrations resulted in a decrease in hCMEC/D3 cell number. Neither TNF-α alone nor TNF-α and IFN-γ for 24 h reduced cell numbers to a significant extent at any dose assessed (data not shown). Our results suggest that cytokine-induced increases in permeability are not related to decreased cell numbers during the experimental period tested.

We then investigated whether JNK signaling was involved in the cytokine-induced increase in effector caspases. We observed that the TNF-α–increased caspase-3/7 activation (10 ng/ml) at 24 h was partially prevented when hCMEC/D3 cells were pretreated with TAT-TI-JIP153–163 (Fig. 5). Additionally, TNF-α–mediated JNK and caspase-3 activity has been shown to depend on PKC signaling in endothelial cells (35). Pretreatment of hCMEC/D3 cells with Bis-1 almost completely abolished TNF-α–induced effector caspase activity (Fig. 5). Similar results were observed when the cells were preincubated with TAT-TI-JIP153–163 or Bis-1 followed by stimulation with 10 ng/ml TNF-α and IFN-γ for 24 h (Fig. 5). Taken together, these results suggest that 10 ng/ml TNF-α

FIGURE 4. Apoptosis is induced in hCMEC/D3 cells with high concentrations of cytokines. (A) Caspase-3 and caspase-7 activity was assessed by analyzing the luminescence emitted by a luminogenic caspase-3/7 substrate. Luminescence is proportional to the amount of effector caspase activity present in the cells. Data were normalized to unstimulated hCMEC/D3 cells. Staurosporin (500 nM, 6 h) was used as a positive control. Results are representative of two independent experiments with triplicate determinations. Error bars represent SEM. Data from one representative experiment are shown (n = 2, in triplicate). *p < 0.05, **p < 0.001 (compared with unstimulated cells), ***p < 0.001 (compared with TNF-α–treated cells). (B) Cytokine-induced apoptosis was measured by determining the percentage of cells positive for annexin V compared to unstimulated cultures. Results are representative of three to four independent experiments with duplicate determinations. Error bars represent SEM (n = 4). *p < 0.05 (compared with nonstimulated cells). (C) Cytokine-induced apoptosis was analyzed by assessing nuclear fragmentation using a TUNEL assay. hCMEC/D3 cells were grown to confluence, stimulated with cytokines at the concentration indicated, and the number of TUNEL+ cells per mm² was determined and normalized to unstimulated hCMEC/D3 cells. Error bars represent SEM (n = 4). **p < 0.01, ***p < 0.001 (compared with nonstimulated cells), *p < 0.05 (compared with TNF-α–treated cells).
alone or in combination with IFN-γ induced an increase in caspase-3/7 activity via the PKC and JNK signaling pathways. Pretreatment with pan-PKC inhibitor almost completely abolished the increase in caspase-3/7 activity. Error bars represent SEM. Data from one representative experiment are shown (n = 2 in triplicate). *p < 0.05, **p < 0.01, ***p < 0.001 (compared with vehicle non-cytokine-stimulated cells), #p < 0.01, ##p < 0.001 (compared with cytokine-treated cells in the absence of inhibitor [vehicle only]).

**FIGURE 5.** Cytokine-induced effector caspase activity was prevented by JNK and PKC inhibitors. Confluent monolayers of hCMEC/D3 cells were pretreated with TAT-TI-JIP (33–163) (JNK inhibitor) or Bis-1 (pan-PKC inhibitor) inhibitors for 40 min before stimulation with either TNF-α alone or in combination with IFN-γ (10 ng/ml) for 24 h. The cells were analyzed for caspase-3/7 activity by measuring luminescence emitted by an effector caspase substrate. JNK inhibitor partially reversed both TNF-α and TNF-α and IFN-γ–induced increases in effector caspase activation. Pretreatment with pan-PKC inhibitor almost completely abolished the increase in caspase-3/7 activity. Effectors were measured by ELISA. Data are represented as fold change compared to control. *p < 0.05, **p < 0.01, ***p < 0.001 (compared with vehicle non-cytokine-stimulated cells), $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (compared with cytokine-treated cells in the absence of inhibitor [vehicle only]).

Caspase-3 and caspase-9 disrupt junctional complexes in hCMEC/D3 cells

Next, we investigated whether caspase signaling was involved in cytokine-induced increase in paracellular permeability in the absence of apoptosis. Using low doses (1 ng/ml) of combination of cytokines, the specific caspase-3 inhibitor Z-DEVD-FMK and the specific caspase-9 inhibitor Z-LEHD-FMK partially prevented TNF-α and IFN-γ–induced leakage of tracer between the luminal and abluminal spaces after 24 h of stimulus (Fig. 6A). Unlike caspase-3 and caspase-9 inhibitors, caspase-8 inhibitor Z-IETD-FMK did not affect the cytokine-induced brain endothelial paracellular leakage (Fig. 6A).

Because 1 ng/ml cytokines did not induce an increase in the number of hCMEC/D3 cells undergoing apoptosis (Fig. 4B, 4C), we then investigated whether increased paracellular permeability might be related to changes in the organization of TJs and AJs by immunofluorescence. Both ZO-1 and VE-cadherin staining in untreated monolayers formed a continuous peripheral belt at the cell–cell junctions (Fig. 6B, 6D, top left). A combination of 1 ng/ml TNF-α and IFN-γ for 24 h induced a marked disruption of both ZO-1 and VE-cadherin staining in the junctional belt without inducing changes in the total protein (Fig. 6C, 6E). Pretreatment with caspase-3 inhibitor partially prevented cytokine-induced disruption of ZO-1 (Fig. 6B) and VE-cadherin (Fig. 6D) compared with cells pretreated with caspase inhibitor negative control. Similar results were observed using a caspase-9 inhibitor (data not shown). Together, these results suggest that caspase-3 signaling is activated after stimulation with 1 ng/ml TNF-α and IFN-γ for 24 h leading to changes in the continuity of junctional complexes and, subsequently, to increased paracellular permeability observed in hCMEC/D3 cells.

**Discussion**

BBB breakdown is associated with BEC dysfunction in neuroinflammatory diseases such as MS and cerebral infections. Experimental observations have shown that administration of TNF-α to the cerebral microcirculation induces an increase in BBB permeability in vivo (38, 39). To investigate the molecular events underlying cerebrovascular barrier breakdown during inflammation, several in vitro models have been developed (16, 22, 40, 41).

In this study, we show that TNF-α alone or in combination with IFN-γ induced an increase in paracellular permeability in a dose- and time-dependent manner. The BEC barrier breakdown at high concentrations of cytokines correlated with the activation of effector caspases via JNK and PKC signaling pathways and induced an increase of apoptotic cells. We also demonstrate that at low concentration of cytokines, caspase-9 and caspase-3 inhibitors partially prevented disruption of junctional complex molecules and increased paracellular permeability in the absence of apoptosis.

TNF-α is a pleiotropic cytokine and exerts its biological activity by virtue of formation of homotypic, oligomeric clustering of its receptors TNFR1 and TNFR2, respectively (42, 43). Although the specific cellular response to TNF-α is largely determined at the level of intracellular signal cascades, receptor subtype and relative density play a role in modulating responsiveness, too. Recently, it was shown that a low percentage of primary human microvascular BECs expressed TNFR1 and TNFR2 under basal conditions (14), which is in agreement with our analysis of hCMEC/D3 cells. Similarly, the interaction of IFN-γ with its receptor, IFNγR, leads to modulation of gene expression in human BECs (44) and activation (14, 45–47).

The mechanisms by which cytokines induce alterations in the gate and fence function of cerebrovascular endothelial cells are largely unknown. Few studies have proposed that cytokines might induce endothelial barrier dysfunction via apoptosis (17, 48–51). However, one report suggests that primary cultures of human BECs are resistant to cell death mediated by TNF-α (18), although this resistance to apoptosis is not observed in primary cultures of BECs of other species (13, 48). These differences in the apoptotic response to cytokines might be associated with cytokine concentrations, time of exposure, cytokine activity, and intrinsic differences among cell types and species. In this study, we observed that hCMEC/D3 cells treated with high concentrations of TNF-α alone or in combination with IFN-γ were associated with increased number of annexin V+ and TUNEL+ cells and activation of caspase-3/7, and these effects appeared to be mediated by JNK and PKC pathways. Reports have demonstrated that sustained JNK activation and increased AP-1 activity evoke apoptosis via mitochondrial cytochrome c release and activation of caspase-3 and caspase-7 (36, 52). More recently, a novel mechanism was demonstrated by which TNF-α induced activation of the PKCζ shorter form, named catalytic domain of PKCζ, which enhanced JNK and caspase-3 activation driving endothelial cells into cell death by apoptosis (35). However, other studies have provided evidence that cells can develop protective pathways during stress conditions capable of buffering apoptosis progression in the presence of caspase activation (53, 54). Yet, when the cytokine stimuli are sustained and intense, as it happens during autoimmune diseases, antiapoptotic mechanisms might fail to protect cells from death (55).

Notably, several reports suggest that caspase-3 is not only an important regulator of cell death by inducing apoptosis but also...
plays important roles in inflammation and physiological processes that do not involve cell death (20, 56, 57). In this study, we have observed that hCMEC/D3 cells exposed to low cytokine concentrations increase effector caspase activation and ZO-1 delocalization in the absence of apoptosis. Indeed, Zehendner and colleagues (21) recently reported that in a model of the neurovascular unit, caspase-3 is strongly activated after 30 min of oxygen and glucose deprivation leading to ZO-1 and claudin-5 disorganization and barrier dysfunction associated with minimal DNA fragmentation. Disassembly of ZO-1 from the endothelial cell borders was not associated with changes in the total protein concentration, suggesting that TNF-α and IFN-γ induced redistribution of ZO-1 rather than protein degradation. It has been observed by immunostaining that disruption of ZO-1 at the cell-to-cell contact appears to correspond to the greatest zones of stress fiber formation, raising the possibility that cytoskeletal rearrangement might be responsible for ZO-1 staining fragmentation from endothelial cell borders (28).

Additionally, caspase effectors have numerous substrates that regulate cytoskeletal and structural proteins that might be partially contributing to ZO-1 redistribution (58). Indeed, in primary cultures of retinal endothelial cells, TNF-α induces changes in protein levels of ZO-1 and claudin-5 as early as 6 h after treatment (59), and long-term exposure to TNF-α correlates with redistribution of junctional adhesion molecule, occludin, and ZO-1 proteins from the cell–cell junction (30). In addition, treatment of peripheral microvascular endothelium with TNF-α and IFN-γ results in focal loss of VE-cadherin–mediated intercellular adhesion in areas of endothelial barrier breakdown (12). Efforts to identify the molecular mechanisms involved in TNF-α–induced redistribution of VE-cadherin from the cell–cell junction have identified reorganization of the actin cytoskeleton via the hierarchical cascade activation of GTP-binding proteins cdc42, Rac, and Rho (60) and tyrosine phosphorylation mediated by JNK activation (61) as important regulators of AJ organization. Another mechanism has involved the cleavage of β-catenin via effector caspases resulting in the disappearance of VE-cadherin from the cell surface in endothelial cells (62). Similar to our results showing ZO-1 redistribution after stimulus with cytokines, disassembly of VE-cadherin from the hCMEC/D3 cell border was not correlated with changes in total protein content. Pretreatment with caspase-3 and caspase-9 inhibitors, but not with caspase-8 inhibitor, partially prevented ZO-1 and VE-cadherin delocalization from the hCMEC/D3 cell border after treatment with low concentrations of TNF-α and IFN-γ, an effect that correlates with a small reduction in the cytokine-induced increase in paracellular permeability. Our results suggest that the effect of cytokine-induced caspase activation that results in remodeling of TJs and AJs is small, and other molecular mechanism including reactive oxygen species, metalloproteinases, phospholipase C-γ, Rho GTPase, PKC, and endothelial cytosolic Ca²⁺ signaling (48, 63, 64) might follow an ordered pattern of activation in response to inflammatory stimuli, which might have
a more profound effect in the overall barrier dysfunction observed in hCMC/E3D cells.

It is worth noting that BEC apoptosis may be differentially regulated by local cytokine levels in vivo in areas of focal inflammation as is the case in MS acute lesions and cerebral infarctions. It is tempting to speculate that in MS, infiltrating leukocytes and brain resident cells might create restricted microenvironments in which levels of inflammatory mediators such as proinflammatory cytokines may be much more elevated than those reported in peripheral blood or in cerebrospinal fluid (12, 65, 66). Cytokine levels at the inflammatory cuff might be high enough to induce apoptosis in BEC and gross changes in BBB permeability (4, 9). By contrast, lower levels of cytokines in and around the inflammatory lesions or even in normal-appearing white matter may result in moderate BBB leakage and disassembly of TJ s (4).

In conclusion, we found that a high concentration of TNF-α alone (>10 ng/ml) or in combination with IFN-γ induced hyperpermeability due to caspase-3/7 activation driving hCMC/E3D cells into apoptosis. At levels of cytokines below the threshold of apoptosis induction in BECs, caspase-3 and caspase-9 signaling contributed to changes in the organization of junctional complexes and increased paracellular permeability in human brain endothelium.

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Disclosures

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