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Nitric oxide reduces T lymphocyte adhesion to human brain microvessel endothelial cells via a cGMP-dependent pathway

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Abstract

The entry of lymphocytes into the brain is normally limited by the blood–brain barrier, however, during inflammation prominent lymphocytic infiltration occurs. In this study, we investigated the effects of nitric oxide (NO) on the adhesion of T cells to cultured human brain microvessel endothelial cells. T cell adhesion to unstimulated or tumor necrosis factor-alpha (TNF-α)-treated cells was quantified by counting the number of lymphocytes bound to the monolayer by light microscopy. TNF-α increased T cell adhesion in a time-dependent manner. Incubation of monolayers with NO donors decreased adhesion. This effect was blocked by a guanylyl cyclase inhibitor and mimicked by a cGMP agonist, and was thus dependent on the generation of cGMP. NO did not modulate adhesion molecule expression in the endothelial cells, suggesting an action on the T cells. Pre-treatment of T cells with NO or a cGMP agonist decreased binding to recombinant endothelial adhesion molecules. These findings suggest that NO can modulate the adhesion of T cells to human brain microvessel endothelial cells via a cGMP-dependent mechanism, and may thus regulate lymphocyte traffic during central nervous system inflammation.

Keywords: T cell; Leukocyte; cGMP-dependent protein kinase; Adhesion molecule; Tumor necrosis factor-alpha; E-selectin; ICAM-1 (intercellular adhesion molecule 1)

1. Introduction

Disruption of the blood–brain barrier is a prominent feature of central inflammatory disease and trauma. Although the factors responsible are not fully understood, evidence suggests that inflammatory mediators such as cytokines produced at the inflammatory site are at least partly responsible for changes in vascular permeability and leukocyte infiltration. Nitric oxide (NO) has also been suggested to play a role in regulating both vascular permeability and leukocyte infiltration (Kubes et al., 1991; Kubes and Granger, 1992; Hickey and Kubes, 1997; Hinder et al., 1997). NO is produced from arginine by various isoforms of nitric oxide synthase, and activates the enzyme soluble guanylyl cyclase in target cells to produce cGMP, which in turn, can activate cGMP-dependent protein kinase. In animal studies, NO generators reduced tissue damage by reducing leukocyte infiltration and reducing the drop in endothelial permeability (Gauthier et al., 1994; Al-Naemi and Baldwin, 1999; Johnston et al., 1999; Al-Naemi and Baldwin, 2000; Schutte et al., 2001; Xu et al., 2001). Inhibition of nitric oxide synthase increased leukocyte adhesion to and migration across feline mesenteric venules (Kubes et al., 1991). The leukocyte adhesion molecule CD11/CD18 was up-regulated by nitric oxide inhibitors (Kubes et al., 1991). Guanylyl cyclase activation appears to play a key step in the inhibition of T cell activation by NO (Bingisser et al., 1998).

We have developed an in vitro model of the blood–brain barrier using primary cultures of human brain microvessel

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endothelial cells which can be used to study interactions with leukocytes (Dorovini-Zis et al., 1991; Shukaliak-Quandt et al., 2003). These cultures express endothelial NOS as well as platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) constitutively and can be induced by cytokines such as TNF-α to upregulate the expression of the adhesion molecules E-selectin, vascular cell adhesion molecule 1 (VCAM-1, CD106) and intercellular adhesion molecule 1 (ICAM-1, CD54) (Wong and Dorovini-Zis, 1995, 1996a,b; Wong et al., 2004a). We have recently found that NO can act through a cGMP-dependent mechanism to decrease the permeability of this in vitro module of the blood–brain barrier (Wong et al., 2004a). Furthermore, we observed that NO modulates the interactions of polymorphonuclear leukocytes with brain microvessel endothelial cells and decreases the binding of the polymorphonuclear leukocytes to the adhesion molecules E-selectin and ICAM-1 (Wong et al., 2004b). T cells also adhere to TNF-α treated brain microvessel endothelial cells through VCAM-1, ICAM-1 and PECAM-1 (Wong et al., 1999). Furthermore, brain microvessel endothelial cells express lymphocyte function-associated antigen 3 (LFA-3, CD58) and provide secondary signals for T cell proliferation (Omari and Dorovini-Zis, 1999). Therefore, in the present study, we examined how T cell adhesion to human brain microvessel endothelial cells can be modulated by NO and the signal transduction pathway involved. We found that NO donors reduced T cell adhesion to these endothelial cells, and this is dependent on cGMP production. Pretreatment of T cells with NO donors or a cGMP agonist reduced binding to recombinant ICAM-1 and PECAM-1. NO and cGMP may thus function in the regulation of T cell infiltration into the central nervous system.

2. Materials and methods

2.1. Human brain microvessel endothelial cell culture

Primary cultures of human brain microvessel endothelial cells were established from brains at autopsy as previously described (Dorovini-Zis et al., 1991; Wong et al., 2004b). The endothelial nature of these cells was confirmed by the positive staining for Factor VIII-related antigen and binding of *Ulex europeaus* agglutinin. Cells were grown on fibronectin coated 96 well plates and cultured in Medium 199 with 10% horse serum. 9–10 day old confluent cultures were used. Several primary cultures from different autopsy brains were utilized.

2.2. T cell isolation

Peripheral blood mononuclear cells were isolated from anti-coagulated peripheral blood of healthy volunteers by centrifugation in Histopaque (Sigma, Oakville, ON). T lymphocytes were separated by passage of PBMC through a "T cell recovery column" (Cedarlane). Monocytes and B lymphocytes were trapped in the column by size and by the action of anti-immunoglobulin antibodies on the column beads, respectively. By this method, greater than 90% of cells eluted were T lymphocytes by fluorescence-activated cell sorting analysis and viability was 99% by trypan blue exclusion test.

2.3. Reagents

Tumor necrosis factor-α (TNF-α) was from Sigma. N^G-nitro-L-arginine methyl ester (L-NAME), 8-Br-cGMP and 1*H*[1,2,4]oxadiazolo[4,3-a]quinoxaliin-1-one (ODQ) from Biomol (Plymouth Meeting, PA). DETA NONOate from Cayman Chemicals (Ann Arbor, MI). Sodium nitroprusside (SNP) from BDH/VWR (Mississauga, ON). cGMP ELISA kit from Biomol. Recombinant human E-selectin from Dr. D. Lyons (Boulder, CO). Recombinant human VCAM-1 from Dr. R. Lobb (Cambridge, MA). Recombinant human ICAM-1 from Dr. M. Dustin (St. Louis, MO). Recombinant human PECAM-1 from Dr. P. Newman (Milwaukee, WI). Anti-ICAM-1 (CA7) from Dr. Rothlein (Ridgefield, CI). Anti-E-selectin and anti-VCAM-1 from Dr. W. Newman (Rockville, MD). Anti-ICAM-1 from Immunocorp (Montreal, PQ). Anti-PECAM-1 from Dr. Albelda (Philadelphia, PA).

2.4. Controls

Monolayers grown in the absence of TNF-α, or in the presence of TNF-α only, as well as monolayers incubated with NO donor solutions prepared 24 h earlier served as controls.

2.5. Adhesion assay

Each adhesion molecule was maximally induced on the brain microvessel endothelial cells by treatment with 100 U TNF-α/ml for 18–24 h (18 h for VCAM-1, 24 h for ICAM-1 and PECAM-1) along with the various donors and inhibitors. T cells (2×10^6 cells/ml) were then added to the wells and incubated with the endothelial cells in the presence of the donors and inhibitors for 30 min at 37 °C. At the end of the incubation period, the supernatants with the nonadherent T cells were removed and the monolayers with the adherent T cells were fixed in 1:1 acetone:ethanol, and stained with the immunoperoxidase technique for leukocyte common antigen. The number of leukocytes bound to the monolayers was determined by counting the number of adherent T cells per mm^2 of the culture dish in 5 fields by light microscopy. In the control assays, the average number of adherent cells was 11/mm^2.

2.6. Adhesion assay to recombinant adhesion molecules

96 well enzyme-linked immunosorbent assay (ELISA) plates (Dynex Corp, Chantilly, VA) were coated with rhE-

selectin at 3 μg/ml in phosphate-buffered saline (PBS), rhVCAM-1 at 10 μg/ml in bicarbonate buffer, anti-ICAM-1 (CA7) antibody at 10 μg/ml followed by rhICAM-1 at 10 μg/ml in bicarbonate buffer, or rhPECAM-1 at 10 μg/ml in borate buffer at 4 °C overnight. Unbound recombinant adhesion molecules were removed by two washes with PBS. T cells were incubated with TNF-α and the donors or inhibitors for 30 min or 24 h at 37 °C. At the end of the incubation period, T cells (2×10^6 cells/ml) were added to the ELISA plates coated with individual adhesion molecules and allowed to adhere for 30 min. After washing to remove nonadherent T cells and fixation with acetone:ethanol, adherent cells were stained with haemotoxylin and the number of adherent T cells counted as above.

2.7. Western blot

T cells were treated with TNF-α as above. 1×10^7 cells were used per lane. Cells were lyzed in 100 mM *p*-amidinophenylmethanesulfonyl fluoride (AMSF) with aprotinin, pepstatin and leupeptin then denatured by boiling in sodium dodecyl sulfate (SDS) for 5 min. Both the pellet and the supernatant were ran together on polyacrylamide minigels (SDS–PAGE) with 5% stacking gel and 10% running gel then transferred to nitrocellulose overnight (Amersham Hybond-C extra). The membrane was blocked for 2 h, incubated the primary antibody overnight, then secondary antibody for 2 h. The protein was detected using the Amersham ECL Western Blotting System (Amersham). Antibodies used include rabbit anti-cGMP dependent protein kinase I at 1:4000 dilution, rabbit anti-cGMP dependent protein kinase II at 1:200 dilution (gifts from Dr. S. Pelech, The University of British Columbia, Vancouver, Canada) and donkey anti-rabbit-HRP at 1:5000 dilution (Amersham).

2.8. cGMP quantification

The amount of cGMP was determined using the cGMP assay kit from Biomol (Plymouth Meeting, PA). Briefly, T cells were treated with TNF, NO donors or inhibitors as above. Cells were then lyzed with 0.1 M HCl and centrifuged. The supernatant containing cGMP was incubated in the ELISA plate along with the anti-cGMP antibody for 2 h with shaking then the p-nitrophenyl phosphate substrate. The optical density was determined at 405 nm and the amount of cGMP in the samples calculated from the cGMP standard curve.

2.9. Cell surface ELISA

TNF-α treated as well as donor or inhibitor-treated endothelial cell monolayers were washed with PBS and fixed in 0.025% glutaraldehyde for 10 min. Following a rinse with PBS and 3 washes with PBS containing 4% normal goat sera and 5% bovine serum albumin, cells were incubated for 60 min. with primary antibody at a final concentration of 2 μg/ml for anti-ICAM-1 (Immunocorp), 5 μg/ml for anti-VCAM-1 and anti-E-selectin, 1:10 dilution for anti-PECAM-1 in carrier buffer containing PBS with 4% normal goat sera and 5% bovine serum albumin. At the end of the incubation period, the monolayers were washed with PBS containing 4% normal goat sera and 5% bovine serum albumin, and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse antibody) at a 1:5000 dilution incarrier buffer for 1 h. Subsequent washes in PBS were followed by incubation in 2 mg/ml *o*-phenylenediamine with 0.015% hydrogen peroxide in 0.1 M PBS for 45 min.. The color development was stopped by the addition of 3 N hydrochloric acid and the absorbance read on a ELISA microtiter plate reader at 490 nm. Controls included cultures incubated in growth media in the absence of donors and inhibitors, and monolayers incubated with normal mouse IgG (Cedarlane) at the same concentration as the primary antibody or carrier buffer instead of the primary antibody.

2.10. Statistics

Data from the assays were examined by analysis of variance (ANOVA). Where significant differences were found, Student's *t*-test was applied.

3. Results

Human brain microvessel endothelial cells were treated with TNF-α with or without the various donors and inhibitors for 18 h and 24 h to maximally upregulate VCAM-1 and ICAM-1, respectively. T cells were then placed on top of the monolayers and allowed to adhere for

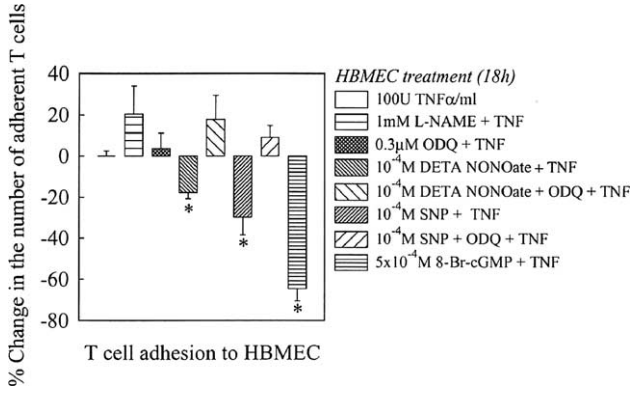

Fig. 1. Quantification of T cell adhesion to human brain microvessel endothelial cells treated with TNF-α and various combinations of the donors and inhibitors. Changes are shown relative to cells receiving only TNF-α for 18 h. Values represent means ± S.E.M. of two experiments, each performed in duplicate wells. *Significant ($P < 0.05$) decrease in adhesion compared to cells treated only with TNF-α. Both NO donors significantly decreased T cell adhesion. This action was reversed by ODQ, suggesting that cGMP is involved. Exogenous 8-Br-cGMP has an even greater effect than NO.

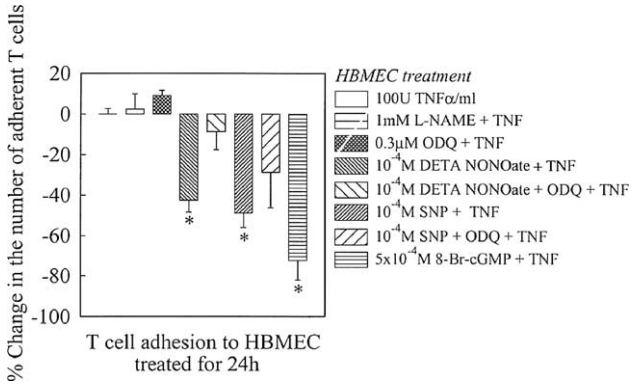

Fig. 2. Quantification of T cell adhesion to human brain microvessel endothelial cells treated with TNF-α and various combinations of the donors and inhibitors. Changes are shown relative to cells receiving only TNF-α for 24 h. Values represent means ± S.E.M. of two experiments, each performed in duplicate wells. *Significant ($P<0.05$) decrease in adhesion compared to cells treated only with TNF-α. Both NO donors significantly decreased T cell adhesion. This action was reversed by ODQ, suggesting that cGMP is involved. Exogenous 8-Br-cGMP has an even greater effect than NO.

30 min. TNF treatment increased adhesion by 7 fold after 18 h and 11 fold after 24 h. Adhesion was also assessed after treatment with the NOS inhibitor L-NAME, the guanylyl cyclase inhibitor ODQ, and the NO donors DETA NONOate and SNP. Changes are shown relative to cells receiving only TNF. The NOS inhibitor L-NAME and the guanylyl cyclase inhibitor ODQ did not have a significant effect on the level of adhesion at 18 h (Fig. 1). Both of the NO donors, DETA NONOate and SNP, significantly decreased adhesion. This was prevented by the guanylyl cyclase inhibitor ODQ. Furthermore, the exogenous cGMP agonist, 8-Br-cGMP was even more effective than the NO donors. After 24 h of treatment, L-NAME and ODQ remained without effect (Fig. 2), while both NO donors and 8-Br-cGMP reduced adhesion. ODQ prevented the action of the NO donors.

To examine whether this modulation of T cell adhesion involves an effect on the level of E-selectin, VCAM-1,

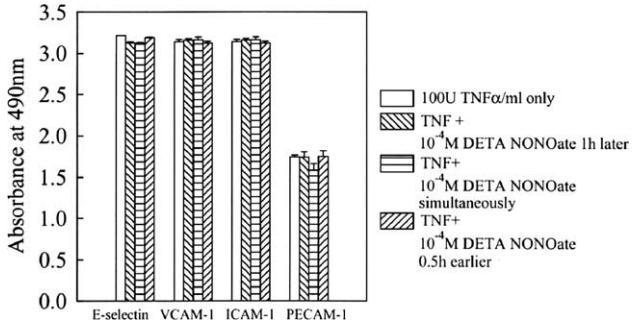

Fig. 3. Quantification of adhesion molecule expression by cell surface ELISA. Human brain microvessel endothelial cells were treated with TNF-α for 4 h to maximally upregulate E-selectin, and 24 h for ICAM-1. NO treatment did not affect the level of adhesion molecule expressed by the endothelial cells. Values represent means ± S.E.M. of two experiments each performed in triplicate culture wells.

Table 1
Quantification of the level of cGMP in T cells by ELISA after 30 min of treatment

Treatment	[cGMP] (pmol/ml)
Untreated	0.382 ± 0.022
100U TNFα/ml	0.445 ± 0.046
TNF + 0.3 mM ODQ	0.333 ± 0.046
TNF + 10^{-4} M DETA NONOate	1.163 ± 0.037*
TNF + DETA NONOate + ODQ	0.302 ± 0.023
TNF + 10^{-4} M SNP	2.031 ± 0.026*
TNF + SNP + ODQ	0.353 ± 0.037

TNF-α did not significantly change the level of cGMP in T cells. Both NO donors significantly increased the level of cGMP. This is inhibited by the guanylyl cyclase inhibitor ODQ.
* Significant ($P<0.05$) change in the level of cGMP compared to untreated cells.

PECAM-1 and ICAM-1 expressed on the endothelial cells, cell surface ELISAs were performed. The endothelial cells were treated with TNF for 4 h, which doubles E-selectin expression (Wong and Dorovini-Zis, 1996b), 18 h to double VCAM-1 expression (Wong and Dorovini-Zis, 1995) or 24 h to increase ICAM-1 (Wong and Dorovini-Zis, 1992) and PECAM-1 (Wong and Dorovini-Zis, 1996a). 10^{-4} M DETA NONOate was added 1 h after the TNF, at the same time as the TNF or 30 min before the TNF. None of the NO treatments affected the ability of TNF to enhance the expression of E-selectin, VCAM-1, PECAM-1 or ICAM-1 determined by ELISA (Fig. 3). It is possible that the NO and cGMP are affecting some other function of the endothelial cells or they might be acting on the T cells.

We therefore examined the T cells. First the levels of cGMP were assessed. TNF did not significantly increase the level of cGMP in T cells. In contrast, both NO donors increased cGMP levels (Table 1). This was inhibited by the

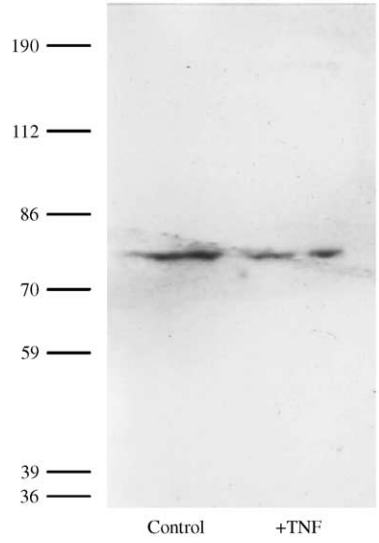

Fig. 4. Detection of cGMP-dependent protein kinase by western blot. Type I cGMP dependent protein kinase is present in unstimulated and TNF-α treated T cells.

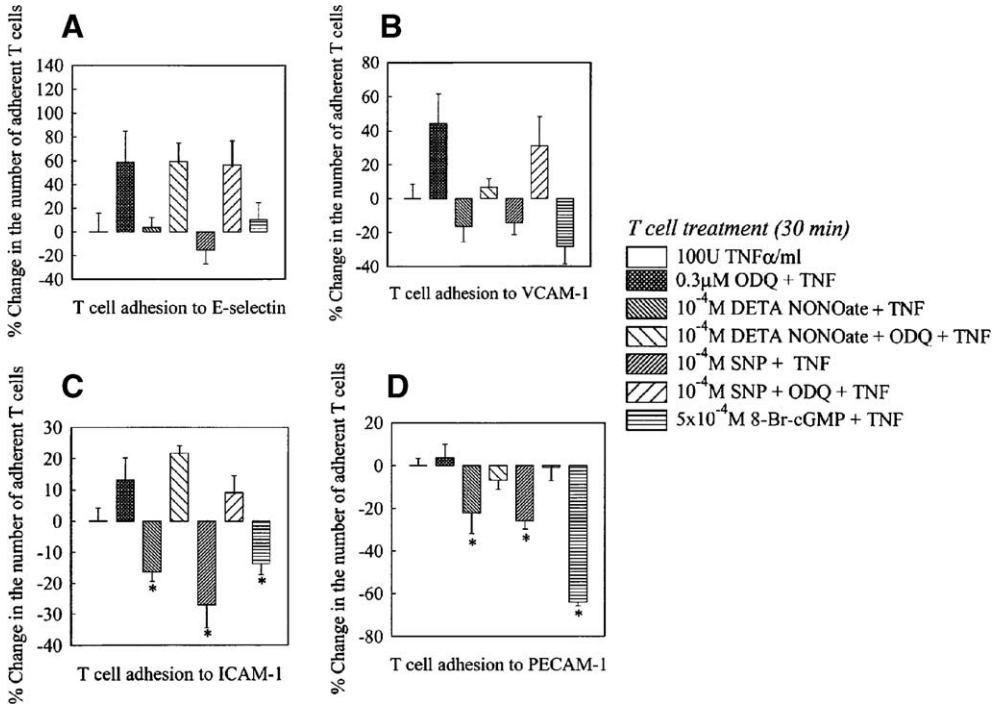

Fig. 5. Quantification of T cell adhesion to recombinant adhesion molecules after T cell treatment with TNF-α and various donors and inhibitors for 30 min. Values represent means ± S.E.M. of three experiments, each performed in duplicate wells. *Significant ($P<0.05$) decrease in adhesion. None of the treatments affected adhesion to E-selectin or VCAM-1. Both NO donors decreased adhesion to ICAM-1 and PECAM-1. ODQ reversed this effect, while 8-Br-cGMP had a similar effect as the NO donors.

guanylyl cyclase inhibitor ODQ. Further, we also detected Type I cGMP-dependent protein kinase by Western blot in both unstimulated and TNF treated T cells (Fig. 4).

Since T cells possess the NO receptor soluble guanylyl cyclase, and the cGMP-dependent protein kinase, we proceeded to examine their adhesion to recombinant E-

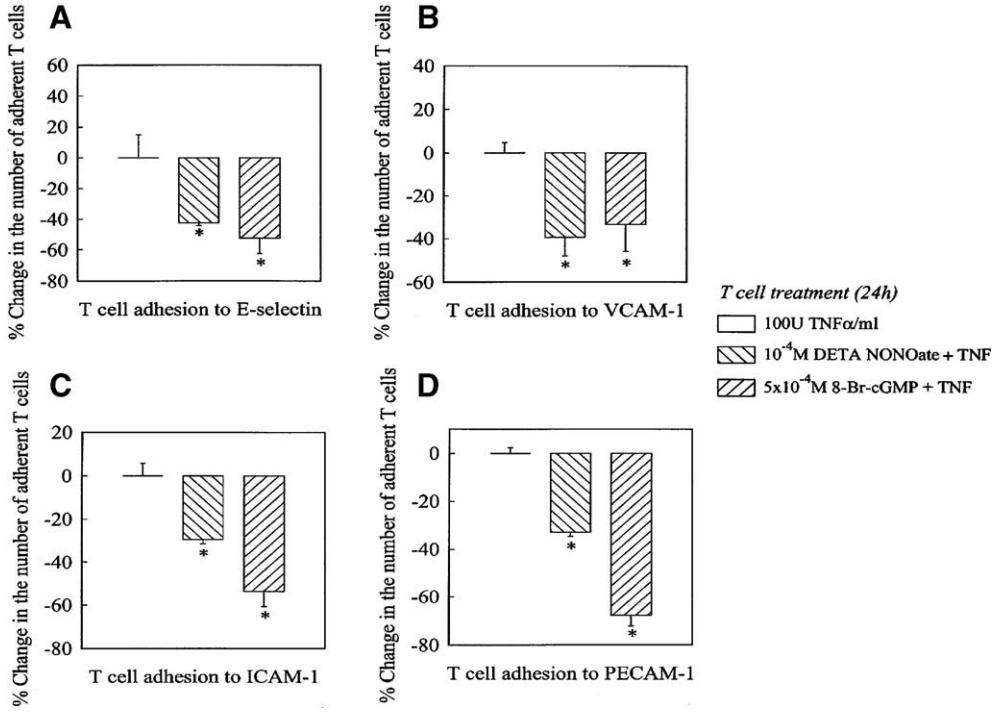

Fig. 6. Quantification of T cell adhesion to recombinant adhesion molecules after T cell treatment with TNF-α and various donors and inhibitors for 24 h. Values represent means ± S.E.M. of three experiments, each performed in duplicate wells. *Significant ($P<0.05$) decrease in adhesion. Treatment with DETA NONOate and 8-Br-cGMP consistently decreased T cell adhesion to all the adhesion molecules.

selectin, VCAM-1, ICAM-1 and PECAM-1 in ELISA plates. Very few T cells adhere to E-selectin. TNF treatment of T cells for 30 min increased their adhesion to E-selectin by 50%. None of the inhibitors or donors significantly changed this (Fig. 5A). 30 min of TNF treatment increased T cell adhesion to VCAM-1 by 60%. None of the treatments significantly affected this adhesion (Fig. 5B). Adhesion to ICAM-1 was increased by 30% after 30min of TNF treatment. Both NO donors significantly decreased the level of adhesion (Fig. 5C). ODQ reversed this effect. 8-Br-cGMP mimicked the action of NO. Adhesion to PECAM-1 was increased 2 fold by 30 min of TNF treatment. Both NO donors significantly decreased this interaction (Fig. 5D). The action of NO was inhibited by ODQ, while 8-Br-cGMP had a even greater effect than the NO donors.

The long term effects of NO and 8-Br-cGMP were assessed next. 24 h of TNF treatment increased T cell adhesion to E-selectin by 2 fold. 24 h of DETA NONOate significantly decreased adhesion. 8-Br-cGMP was slightly more effective than NO (Fig. 6A). TNF treatment for 24 h increased T cell adhesion to VCAM-1 by 80%. Both DETA NONOate and 8-Br-cGMP are effective in decreasing adhesion after 24 h (Fig. 6B). Adhesion to ICAM-1 was increased by 20% after TNF treatment. 8-Br-cGMP is slightly more effective than DETA NONOate in reducing this adhesion (Fig. 6C). TNF treatment for 24 h increased T cell adhesion to PECAM-1 by 30%. 8-Br-cGMP is more effective in reducing this adhesion than DETA NONOate (Fig. 6D).

4. Discussion

Inflammatory reactions are characterized by extravasation and infiltration of tissue by leukocytes. The blood–brain barrier, formed by the cerebral endothelial cells, normally impedes the entry of inflammatory cells into the brain. Previous work from this laboratory has shown that E-selectin, VCAM-1, and ICAM-1 can be upregulated in human brain microvessel endothelial cells by lipopolysaccharides and cytokines such as TNF-α (Wong and Dorovini-Zis, 1995, 1996b). E-selectin is maximally expressed at 4 h, VCAM-1 between 12 and 24 h, and ICAM-1 at 24 h. PECAM-1 is constitutively expressed by the endothelial cells (Wong and Dorovini-Zis, 1996a). Human peripheral blood T cells adhere to the brain microvessel endothelium via VCAM-1 and ICAM-1 (Wong et al., 1999). The latter also provides secondary signals for T cell proliferation at least partly through CD58 (Omari and Dorovini-Zis, 1999).

The inhibition of T cell activation is mimicked by cGMP agonists and prevented by guanylyl cyclase inhibitors, implicating soluble guanylyl cyclase in this pathway (Bingisser et al., 1998). T cells express high levels of a cytoplasmic guanylyl cyclase activity (Deviller et al., 1975; Takemoto et al., 1982; Cille et al., 1983). Both human endothelial cells (Draijer et al., 1995) and T cells (Fischer et al., 2001; Lossos et al., 2003) express cGMP-dependent protein kinase I. Incubation of human T cells with the NO donor, sodium nitroprusside, or cGMP agonists activate this kinase leading to phosphorylation of vasodilator-stimulated phosphoprotein and stimulation of various mitogen-activated protein kinases (Fischer et al., 2001). Upon anti-CD3 stimulation this leads to inhibition of interleukin 2 release and inhibition of cell proliferation (Fischer et al., 2001). IFNγ induces NO production by dendritic cells which leads to apoptosis in autoreactive T cells (Xu et al., 1999). The expression of cGMP-dependent protein kinase is lost during in vitro culturing of primary T cells and is not detectable in transformed T cell lines (Fischer et al., 2001).

The literature regarding the role of NO in the immune system has been and remains controversial, partly due to species variations in regulation (Schneemann and Schoedon, 2002). Thus, it is important to study human as well as animal tissues and tissues from various organ systems. As far as we know, there has been no study on the effect of NO on the interactions between human brain microvessel endothelial cells and T cells. In the present study, we examined whether the interaction between T cells and the blood-brain barrier can be modulated by NO and the mechanism and signal transduction pathway involved. TNF-α treatment of these endothelial cells for 18 or 24 h, or T cells for 30 min or 24 h significantly increases T cell adhesion to EC. NO reduces T cell adhesion by a pathway involving cGMP production and the activation of cGMP-dependent protein kinases.

A few studies have shown that NO donors reduce monocyte adhesion. SNP reduced cell adhesion molecule expression on human saphenous vein endothelial cells and reduced monocyte adhesion (De Caterina et al., 1995). Similar effects have been observed with a variety of NO donors: GSNO, spermine NONOate, SP/W 3672 and SP/W 5186 (Zampolli et al., 2000). Comparable to our results, the NO donors, SNP and GSNO had no effect on the expression of VCAM-1, ICAM-1 and E-selectin on SGHEC-7, a cell line derived from human umbilical cord endothelial cells (Cartwright et al., 1997). A large body of literature, mainly with umbilical cord endothelial cells has implicated reduced cell adhesion molecule expression in these effects. The NO donors DETA-NO and SIN-1 both reduced upregulation of VCAM-1, ICAM-1 and E-selectin by TNF-α, IL-1β and lipopolysaccharides (Biffl et al., 1996; Khan et al., 1996). IL-1β also upregulated these adhesion molecules in human saphenous vein endothelial cells. SNP, SIN-1, GSNO, spermine NONOate and other NO donors inhibited the expression of these adhesion molecules. However, some NO donors, nitroglycerine and isosorbide dinitrate were ineffective possibly due to their short half-life (De Caterina et al., 1995; Zampolli et al., 2000). Similar effects have been observed in microvessels. ICAM-1 expression induced by hypoxia, reoxygenation in rat cornea microvascular cells is suppressed by *S*-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP) (Kupatt et al., 1997),

while VCAM-1 induced by TNF-α is reduced by DETA-NO (Khan et al., 1996). The present study involved human brain microvessel endothelial cells which have not been studied before. The negative results observed on adhesion molecule expression may be due to differences between tissues from different species and organ systems, compounded by the concentration of NO donors used, which is at the lower range of that found to be effective in the literature. Although the expression of cell adhesion molecules is unaffected by a NO donor in our other studies, other functions of these endothelial cells may be modified. A number of other endothelial cell functions have been shown to be affected by NO. GSNO, spermine NONOate and other NO donors reduced MHCII expression on human saphenous vein endothelial cells induced by interferon-γ (IFNγ) (Zampolli et al., 2000). SIN-1 reduced TNF-α induced monocyte chemoattractant protein-1 (MCP-1) secretion by umbilical cord endothelial cells, and the subsequent chemotaxis of monocytes. A reduction in NF-κB activation is suggested. Conversely, blockage of NOS by L-NG-nitroarginine (L-NNA) increases MCP-1 mRNA protein secretion (Zeiher et al., 1995; Rossig et al., 2000). In agreement, activated protein kinase C reduces eNOS in umbilical cord endothelial cells and induced MCP-1 production. T cells activated in the presence of alveolar macrophage cannot proliferate despite the presence of IL-2. An NO effect was proposed since SNAP and 8-Br-cGMP produced the same effects (Bingisser et al., 1998). L-NMA, on the other hand, had the opposite effect. The mechanism involves reduced tyrosine phosphorylation of Jak 3 and STATS mediated by NO and activation of guanylyl cyclase.

In addition to actions on the endothelium, the adhesive properties of T cells are also modulated by NO. NO donors and 8-Br-cGMP significantly reduced T cell adhesion to ICAM-1 and PECAM-1 after 30 min of treatment, and showed a similar, though not statistically significant trend on adhesion to VCAM-1. The production of cGMP is required and the activation of cGMP-dependent protein kinases is partly involved. Both of these steps in the NO signal transduction pathway: the production of cGMP after NO treatment and the expression of cGMP dependent protein kinase I are observed in T cells. This effect does not appear to be due to a toxic action on of NO on the T cells, since the reduction in adhesion was prevented by the guanylyl cyclase inhibitior ODQ. This is consistent with the literature which described guanylyl cyclase activity in both normal and leukemic lymphocytes (Takemoto et al., 1982). The guanylyl cyclase activity in human T cells is mainly found in the soluble fraction (Deviller et al., 1975; Cille et al., 1983). SNP did not affect L-selectin expression on lymphocytes (Stibenz et al., 1996), but it has not been shown whether other receptors involved in adhesion is altered. Longer term (24 h) treatment causes an inhibition of T cell adhesion to E-selectin and VCAM-1 as well as ICAM-1 and PECAM-1, but only with DETA NONOate which has a long half-life (20 h) and not SNP (half-life of a few minutes).

A number of animal studies support the contention that NO has the potential to modulate T cell–blood–brain barrier interactions. During the incipient phase of experimental autoimmune encephalomyelitis in Lewis rats, IL-4 treatment activates dendritic cells to produce increased IFNγ and IL-10. The former increases NO production. This dendritic cell derived NO increases apoptosis of autoreactive T cells. This feedback was proposed to be partly responsible for reduced clinical signs (Xu et al., 1999). In another study, SIN-1 administration reduced clinical symptoms as well as macrophage and CD4+T cell infiltration into the central nervous system, at least partly via increased apoptosis (Xu et al., 2001). Related to this, there is a reduction in MHCII, B7-1, B7-2 expression, antigen presentation and mitogen induced proliferation responses. Thus, NO and components of its signal transduction pathway may modulate the adhesive interactions between the cerebral endothelium and circulating T cells in various human central nervous system inflammatory disorders. They may therefore be considered a potential therapeutic agent for multiple sclerosis and similar conditions.

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References

Al-Naemi, H., Baldwin, A.L., 1999. Nitric oxide: role in venular permeability recovery after histamine challenge. Am. J. Physiol. 277, H2010–H2016.

Al-Naemi, H., Baldwin, A.L., 2000. Nitric oxide protects venules against histamine-induced leaks. Microcirculation 7, 215–223.

Biffl, W.L., Moore, E.E., Moore, F.A., Barnett, C., 1996. Nitric oxide reduces endothelial expression of intercellular adhesion molecule (ICAM)-1. J. Surg. Res. 63, 328–332.

Bingisser, R.M., Tilbrook, P.A., Holt, P.G., Kees, U.R., 1998. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. J. Immunol. 160, 5729–5734.

Cartwright, J.E., Whitley, G.S., Johnstone, A.P., 1997. Endothelial cell adhesion molecule expression and lymphocyte adhesion to endothelial cells: effect of nitric oxide. Exp. Cell Res. 235, 431–434.

Cille, Y., Deviller, P., Betuel, H., 1983. Guanylate cyclase activity of human lymphocytes from peripheral blood, thymus, and tonsils. A comparative study. Enzyme 29, 86–92.

De Caterina, R., Libby, P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone Jr., M.A., Shin, W.S., Liao, J.K., 1995. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. J. Clin. Invest. 96, 60–68.

Deviller, P., Cille, Y., Betuel, H., 1975. Guanyl cyclase activity of human blood lymphocytes. Enzyme 19, 300–313.

Dorovini-Zis, K., Prameya, R., Bowman, P.D., 1991. Culture and characterization of microvascular endothelial cells derived from human brain. Lab. Invest. 64, 425–436.

Draijer, R., Vaandrager, A.B., Nolte, C., de Jonge, H.R., Walter, U., van Hinsbergh, V.W., 1995. Expression of cGMP-dependent protein kinase I and phosphorylation of its substrate, vasodilator-stimulated phosphoprotein, in human endothelial cells of different origin. Circ. Res. 77, 897–905.

Fischer, T.A., Palmetshofer, A., Gambaryan, S., Butt, E., Jassoy, C., Walter, U., Sopper, S., Lohmann, S.M., 2001. Activation of cGMP-dependent protein kinase Ibeta inhibits interleukin 2 release and proliferation of T cell receptor-stimulated human peripheral T cells. J. Biol. Chem. 276, 5967–5974.

Gauthier, T.W., Davenpeck, K.L., Lefer, A.M., 1994. Nitric oxide attenuates leukocyte–endothelial interaction via P-selectin in splanchnic ischemia–reperfusion. Am. J. Physiol. 267, G562–G568.

Hickey, M.J., Kubes, P., 1997. Role of nitric oxide in regulation of leucocyte–endothelial cell interactions. Exp. Physiol. 82, 339–348.

Hinder, F., Booke, M., Traber, L.D., Traber, D.L., 1997. Nitric oxide and endothelial permeability. J. Appl. Physiol. 83, 1941–1946.

Johnston, B., Gaboury, J.P., Suematsu, M., Kubes, P., 1999. Nitric oxide inhibits microvascular protein leakage induced by leukocyte adhesion-independent and adhesion-dependent inflammatory mediators. Microcirculation 6, 153–162.

Khan, B.V., Harrison, D.G., Olbrych, M.T., Alexander, R.W., Medford, R.M., 1996. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. Proc. Natl. Acad. Sci. U. S. A. 93, 9114–9119.

Kubes, P., Granger, D.N., 1992. Nitric oxide modulates microvascular permeability. Am. J. Physiol. 262, H611–H615.

Kubes, P., Suzuki, M., Granger, D.N., 1991. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc. Natl. Acad. Sci. U. S. A. 88, 4651–4655.

Kupatt, C., Weber, C., Wolf, D.A., Becker, B.F., Smith, T.W., Kelly, R.A., 1997. Nitric oxide attenuates reoxygenation-induced ICAM-1 expression in coronary microvascular endothelium: role of NFkappaB. J. Mol. Cell. Cardiol. 29, 2599–2609.

Lossos, I.S., Czerwinski, D.K., Wechser, M.A., Levy, R., 2003. Optimization of quantitative real-time RT-PCR parameters for the study of lymphoid malignancies. Leukemia 17, 789–795.

Omari, K.I., Dorovini-Zis, K., 1999. Expression and function of lymphocyte function associated antigen-3 (LFA-3) at the blood–brain barrier. Cell. Mol. Biol. (Noisy-le-grand) 45, 25–35.

Rossig, L., Haendeler, J., Hermann, C., Malchow, P., Urbich, C., Zeiher, A.M., Dimmeler, S., 2000. Nitric oxide down-regulates MKP-3 mRNA levels: involvement in endothelial cell protection from apoptosis. J. Biol. Chem. 275, 25502–25507.

Schneemann, M., Schoedon, G., 2002. Species differences in macrophage NO production are important. Nat. Immunol. 3, 102.

Schutte, H., Lockinger, A., Seeger, W., Grimminger, F., 2001. Aerosolized PGE1, PGI2 and nitroprusside protect against vascular leakage in lung ischaemia–reperfusion. Eur. Respir. J. 18, 15–22.

Shukaliak-Quandt, J., Wong, D., Dorovini-Zis, K., 2003. Human brain microvessel endothelial cell and leukocyte interactions. Methods Mol. Med. 89, 337–348.

Stibenz, D., Buhrer, C., Laufer, D., Obladen, M., 1996. CD45 engagement induces L-selectin down-regulation. Scand. J. Immunol. 44, 37–44.

Takemoto, D.J., Dunford, C., Vaughn, D., Kramer, K.J., Smith, A., Powell, R.G., 1982. Guanylate cyclase activity in human leukemic and normal lymphocytes. Enzyme inhibition and cytotoxicity of plant extracts. Enzyme 27, 179–188.

Wong, D., Dorovini-Zis, K., 1992. Upregulation of intercellular adhesion molecule-1 (ICAM-1) expression in primary cultures of human brain microvessel endothelial cells by cytokines and lipopolysaccharide. J. Neuroimmunol. 39, 11–21.

Wong, D., Dorovini-Zis, K., 1995. Expression of vascular cell adhesion molecule-1 (VCAM-1) by human brain microvessel endothelial cells in primary culture. Microvasc. Res. 49, 325–339.

Wong, D., Dorovini-Zis, K., 1996a. Platelet/endothelial cell adhesion molecule-1 (PECAM-1) expression by human brain microvessel endothelial cells in primary culture. Brain Res. 731, 217–220.

Wong, D., Dorovini-Zis, K., 1996b. Regulation by cytokines and lipopolysaccharide of E-selectin expression by human brain microvessel endothelial cells in primary culture. J. Neuropathol. Exp. Neurol. 55, 225–235.

Wong, D., Prameya, R., Dorovini-Zis, K., 1999. In vitro adhesion and migration of T lymphocytes across monolayers of human brain microvessel endothelial cells: regulation by ICAM-1, VCAM-1, E-selectin and PECAM-1. J. Neuropathol. Exp. Neurol. 58, 138–152.

Wong, D., Dorovini-Zis, K., Vincent, S.R., 2004a. Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood–brain barrier. Exp. Neurol. 190, 446–455.

Wong, D., Prameya, R., Dorovini-Zis, K., Vincent, S.R., 2004b. Nitric oxide regulates interactions of PMN with human brain microvessel endothelial cells. Biochem. Biophys. Res. Commun. 323, 142–148.

Xu, L., Huang, Y., Yang, J., Van Der Meide, P.H., Levi, M., Wahren, B., Link, H., Xiao, B., 1999. Dendritic cell-derived nitric oxide is involved in IL-4-induced suppression of experimental allergic encephalomyelitis (EAE) in Lewis rats. Clin. Exp. Immunol. 118, 115–121.

Xu, L.Y., Yang, J.S., Link, H., Xiao, B.G., 2001. SIN-1, a nitric oxide donor, ameliorates experimental allergic encephalomyelitis in Lewis rats in the incipient phase: the importance of the time window. J. Immunol. 166, 5810–5816.

Zampolli, A., Basta, G., Lazzerini, G., Feelisch, M., De Caterina, R., 2000. Inhibition of endothelial cell activation by nitric oxide donors. J. Pharmacol. Exp. Ther. 295, 818–823.

Zeiher, A.M., Fisslthaler, B., Schray-Utz, B., Busse, R., 1995. Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells. Circ. Res. 76, 980–986.