Cytokine-induced modulation of cellular adhesion to human cerebral endothelial cells is mediated by soluble vascular cell adhesion molecule-1

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Summary
Tumour necrosis factor-α (TNF-α) has been proposed as one of the key mediators of inflammatory diseases of the CNS such as multiple sclerosis. It has been shown to induce the expression of adhesion molecules which is a prerequisite for the transmigration of immune cells through the blood–brain barrier. We therefore investigated the role of TNF-α in the expression and release of vascular cell adhesion molecule-1 (VCAM-1) in cultures of human cerebral endothelial cells (HCEC) in comparison with peripheral blood mononuclear cells (PBMC). A time- and dose-dependent expression of VCAM-1 and release of soluble VCAM-1 was detected in HCEC but not PBMC. TNF-α-induced release of soluble VCAM-1 was further increased by cotreatment with interferon-β (IFN-β), while IFN-β alone did not affect VCAM-1 expression or the release of soluble VCAM-1. In addition, we observed that preincubation of PBMC with soluble VCAM-1 completely blocked their adhesion to HCEC. In conclusion, the proinflammatory effect of TNF-α on HCEC, which involves the induction of VCAM-1 expression and cellular adhesion, is followed by the consecutive effects of soluble VCAM-1 release in blocking adhesion and downregulating further cellular infiltration. Increasing soluble VCAM-1 release during active inflammation could be another mechanism by which IFN-β treatment exerts protective effects in multiple sclerosis patients.

Keywords: soluble adhesion molecules; multiple sclerosis; blood–brain barrier; VCAM-1; IFN-β

Abbreviations: EAE = experimental autoimmune encephalomyelitis; HCEC = human cerebral endothelial cells; ICAM-1 = intercellular adhesion molecule-1; IFN = interferon; IL = interleukin; PBMC = peripheral blood mononuclear cells; TNF = tumor necrosis factor; VCAM-1 = vascular cell adhesion molecule-1

Introduction
Adhesion molecules are involved in processes of leucocyte attachment and transmigration through the endothelium into areas of inflammation (Springer, 1990). They play a central role in the pathology of autoimmune diseases, e.g. multiple sclerosis (McMurray, 1996). In the model of experimental autoimmune encephalomyelitis (EAE), treatment with a monoclonal antibody to the intercellular adhesion molecule-1 (ICAM-1) suppressed active EAE (Archelos et al., 1993). The vascular cell membrane adhesion molecule (VCAM-1, CD106) is induced by proinflammatory cytokines like tumour necrosis factor-α (TNF-α) on human endothelial cells in a similar manner to ICAM-1 (Osborn et al., 1989). It is also detected on non-vascular cells such as lymphoid dendritic cells (Freedman et al., 1990), macrophages (Rice et al., 1991) and lymphocytes (Leca et al., 1995). Adhesion and activation of lymphocytes is mediated through interaction with its counter-receptor VLA-4 (Burkly et al., 1991).

There is increased expression of VCAM-1 and VLA-4 in active and chronic multiple sclerosis lesions (Cannella and Raine, 1995) as well as in chronic EAE (Steffen et al., 1994). Elevated levels of soluble VCAM-1 in serum and CSF have been found to correlate with disease activity in multiple sclerosis patients as well as with gadolinium-enhancing lesions on MRI, which are regarded as indicators of blood–brain barrier breakdown (Hartung et al., 1995; Rieckmann et al., 1997). The major cellular source of soluble VCAM-1 in multiple sclerosis has not been identified as yet.

TNF-α is regarded as the key proinflammatory cytokine in
the pathological process of multiple sclerosis and is expressed in the inflammatory infiltrate of active and chronic multiple sclerosis plaques (Selmaï et al., 1991). Increased production of TNF-α precedes clinical relapses (Sharief and Hentges, 1991; Rieckmann et al., 1994), enhances blood–brain barrier permeability (Sharief and Thompson, 1992) and can directly induce demyelination (Selmaï and Raine, 1988). Anti-TNF-α strategies have been shown to abrogate EAE (Ruddle et al., 1990).

In contrast, anti-TNF-α treatment in multiple sclerosis patients was followed by increased MRI activity suggesting disease activation (van Oosten et al., 1996). Furthermore, TNF-α is supposed to have anti-inflammatory properties: MOG (myelin oligodendrocyte glycoprotein)-induced EAE causing severe neurological impairment with high mortality in mice lacking the TNF-α gene (Liu et al., 1998), and antigen-induced TNF-α secretion is associated with apoptosis of lymphocytes (Weishaupt et al., 1997).

Interferon-β (IFN-β) was recently introduced into the therapy of multiple sclerosis patients reducing clinical exacerbations and progression of the disease (IFNB Study Group, 1993; Goodkin, 1998). It has been shown to inhibit microglial TNF-α production (Chabot et al., 1997) and IFN-γ-induced MHC (major histocompatibility complex) II expression on macrophages (Ling et al., 1985). Furthermore, IFN-β might modulate the Th1/Th2 balance by reducing human dendritic cell interleukin (IL)-12 production (McRae et al., 1998) and increasing IL-10 release (Porrini et al., 1995). The exact mechanism of IFN-β action in multiple sclerosis is still under debate. Surprisingly, elevated levels of soluble VCAM-1 in sera of multiple sclerosis patients were found not only during relapse but also after the initiation of IFN-β treatment (Calabresi et al., 1997b) and were positively correlated with the early treatment response (Rieckmann et al., 1998).

In our study we compared cultures of human cerebral endothelial cells (HCEC) and peripheral blood mononuclear cells (PBMC) for their ability to upregulate cellular VCAM-1 expression and release of soluble VCAM-1 after treatment with TNF-α. We further investigated whether cotreatment with IFN-β might have a modulatory effect. In addition, the role of soluble VCAM-1 in the cellular adhesion of PBMC to HCEC was investigated under these conditions.

Material and methods

**HCEC**

HCEC were isolated from early post-mortem human adult brains (autopsy <8 h) using a buffered sucrose gradient, as first described by Tontsch and Bauer (Tontsch and Bauer, 1989). In brief, the pia mater and arachnoidea were removed from the cerebrum and white matter was dissected out. Then brain grey matter areas were homogenized and washed several times in buffered sucrose (0.32 M sucrose, 3 mM HEPES, pH 7.4). A short centrifugation (45 s) at 100 g was used for enrichment of human cerebral microvessels in the supernatant of sucrose buffer (Fig. 1A). This step was repeated twice and microvessels were then pelleted by centrifugation at 1000 g for 10 min and the supernatant was discarded. Microvessels were then dissociated with 0.075% collagenase type 1 (Sigma, St Louis, Mo., USA) in Ca²⁺/Mg²⁺-free PBS (phosphate buffered saline) for 15 min at 37°C. After washing cells several times in M199 medium (Biowhittaker, Verviers, Belgium) supplemented with 10% foetal calf serum (Boehringer, Mannheim, Germany), cells were plated on 6-well tissue culture plates (Nunc, Roskilde, Denmark) coated with gelatin (Sigma) in PBS with Ca²⁺/Mg²⁺. M199 medium was supplemented with 20% foetal calf serum, endothelial cell growth supplement (20 µg/ml), heparin (100 µg/ml) (both Sigma) and antibiotics (50 U/ml penicillin, 0.05 mg/ml streptomycin, 0.05 mg/ml gentamicin and 2.5 µg/ml amphotericin) and cultured at 37°C/5% CO₂. The media were changed every other day. During further culturing the foetal calf serum content in the media was reduced to 10%. Confluent HCEC typically showed a cobblestone pattern of growth (Fig. 1B).

Primary cell cultures were further passaged at stages of 70–80% confluence (in general after 5–7 days of culture) by treating adherent cells with 0.1% trypsin/EDTA (Sigma), splitting (1 : 4) and growing on gelatin-coated 24-well tissue culture plates. For further experiments cell cultures from passages 4–8 were used after confirmation of cell purity by immunohistochemistry.

**Experimental conditions**

HCEC were incubated in culture medium with 0, 1 and 10 ng/ml of recombinant TNF-α (Genzyme, Cambridge, Mass., USA) alone or in combination with 8 or 80 U/ml IFN-β (Bettaferon®, Schering, Berlin, Germany) for 24, 72 and 144 h at 37°C/5% CO₂. Supernatants were collected and stored at −80°C for further quantification of soluble VCAM-1 by ELISA (enzyme-linked immunosorbent assay). Cells were washed twice with Ca²⁺/Mg²⁺-free PBS, then detached with 0.1% trypsin/EDTA and washed again with M199/10% foetal calf serum before labelling with *Ulex europeus* lectin or monoclonal antibodies for further evaluation of cell surface expression of VCAM-1 by FACS (fluorescent activated cell sorter) analysis.

**PBMC**

PBMC were isolated from healthy donors by Ficoll density gradient centrifugation of heparinized blood using Hypaque (Nycomed, Oslo, Norway). PBMC at a density of 10⁶ cells/ml were incubated in RPMI 1640 medium (Biowhittaker) supplemented with 10% foetal calf serum and antibiotics for 24, 72 and 144 h with 0, 1 and 10 ng/ml TNF-α, alone or in combination with 8 or 80 U/ml IFN-β (Bettaferon®) at 37°C/5% CO₂. Supernatants were collected and stored at −80°C until further analysis. Cells were washed again with RPMI 1640 before labelling with monoclonal antibodies for further evaluation of the cell surface expression of VCAM-1 by FACS analysis.
Modulation of cellular adhesion

**Immunohistochemistry**

The expression of typical blood–brain barrier-associated molecules and the purity of human cerebral endothelial cell cultures were determined by staining cell cultures with rabbit anti-glucose-transporter-1 (1:100; Chemicon, Temecula, Calif., USA), mouse anti-human P-glycoprotein (multi-drug-resistance-transporter, 1:10; Chemicon), biotinylated lectin from *Ulex europaeus* (1:100; Sigma) (Fig. 2) and rabbit anti-human factor VIII (1:50; Dako, Hamburg, Germany), which are markers specific for HCEC (Dorovini-Zis *et al.*,...
Fig. 2 Immunofluorescence microscopy of HCEC cultured for 72 h without (A, B and C) and with (D, E and F) TNF-α 10 ng/ml. White scale bars represent 100 μm. Double-staining with *U. europaeus* biotin/streptavidin-FITC (A and D) and VCAM-1/Cy3 anti-mouse IgG (B, E) is also shown as an overlay in the same HCEC (C and F).

1991) and were found in more than 95% of cells. To exclude the possibility that cultures had been contaminated with oligodendrocytes, astrocytes and pericytes, they were stained with mouse anti-human galactocerebroside (1 : 50), rabbit anti-glial fibrillary acidic protein (1 : 100; Dako) and mouse anti-smooth muscle antigen (1 : 100; Boehringer Mannheim, Germany) as primary antibodies. A FITC (fluorescein thiocyanate)-labelled streptavidin (1 : 100; Boehringer Mannheim), a FITC-labelled anti-rabbit and anti-mouse IgG antibody (1 : 100; Sigma) was used as secondary antibody. Oligodendrocytes were not detected, and astrocytes and pericytes were seen in less than 5% of HCEC. Before staining, cells were washed twice in PBS/0.2% gelatin and then fixed with ethanol/glacial acetic acid (95/5) for 10 min at -20°C. Cells were then washed twice with PBS/0.2% gelatin and incubated consecutively with primary and
secondary antibodies, diluted in PBS/0.2% gelatin for 60 min at room temperature. Each incubation step was followed by washing twice with PBS/0.2% gelatin. Imaging was performed with an Olympus IX-70 inverted system microscope with Hoffmann modulation contrast and an IX-FLA observation attachment for fluorescence imaging.

**Cell surface expression of VCAM-1 and Ulex europaeus antigen by FACS analysis**

For flow cytometric analysis, HCEC (detached with 0.1% trypsin/EDTA) and PBMC were incubated with primary monoclonal mouse anti-human VCAM-1 IgG antibody (Biosource, Calif., USA) diluted 1 : 10 in PBA (phosphate buffered bovine albumin) buffer (PBS, 0.1% BSA, bovine serum albumin) for 30 min at 4°C. FITC-labelled anti-mouse IgG Fab (1 : 10; Sigma) was used as secondary antibody and cells were again incubated for 30 min at 4°C. Each incubation step was followed by washing cells twice with PBA buffer. An isotype IgG anti-mouse control antibody was used to determine the background signal. In a second set of experiments HCEC were double-stained with *U. europaeus-*biotin/streptavidin-tricolor (0.2 mg/ml; Caltag, Burlingame, Calif., USA) and VCAM-1/FITC anti-mouse as outlined above. Quantitative flow cytometry was performed immediately using a Becton Dickinson FACS.

**Immunofluorescence microscopy**

HCEC were double-stained with biotin-labelled *U. europaeus* lectin and monoclonal mouse anti-human VCAM-1 IgG antibody (1 : 200), FITC-labelled streptavidin (1 : 200; Boehringer Mannheim) and Cy3-anti-mouse IgG (1 : 50; Chemicon) were used as secondary antibodies. Stained HCEC were digitized using a Zeiss Axiophot 2 microscope equipped with a Sony CCD camera interfaced to a PC. For analysis of immunofluorescence, double-stained images were overlaid using Image Pro Plus software 4.0.

**Determination of soluble VCAM-1 by ELISA**

Soluble VCAM-1 in undiluted cell culture supernatants was measured by a commercially available ELISA (R&D Systems, Abingdon, UK). Assays were performed according to the manufacturer’s instructions. In brief, supernatants were added to microwells precoated with a primary monoclonal antibody and incubated for 1 h. Unbound protein was removed by several washing steps. A secondary horseradish peroxidase-conjugated monoclonal antibody was added. After development of a colour reaction with tetramethylbenzidine dye, the absorbance was recorded at 450-nm wavelength with an automated ELISA reader. Quantitative results were obtained from standard curves of recombinant protein standards for soluble VCAM-1 provided by the manufacturer. The inter-assay variability was less than 8%.

**Adhesion assay**

For the adhesion assay confluent monolayers of HCEC were stimulated with 0 or 10 ng/ml TNF-α for 24, 72 and 144 h at 37°C/5% CO₂. PBMC were freshly isolated, incubated with 10 ng/ml TNF-α for 24 h, washed twice with PBS and adjusted to 5 × 10⁶ cells/ml in RPMI 1640. PBMC were then incubated with 5 µl calcAM/ml (calcine-acetoxyethylster; Molecular Probes, Eugene, Oreg., USA) for 30 min at 37°C. CalcAM, a fluorescent dye, is incorporated into living cells, deacetylated and thereby fixed in the cells. PBMC were then washed twice with RPMI 1640 to remove unincorporated dye, and then adjusted to 1 × 10⁶ cells/ml in the medium. The supernatant was carefully removed from the HCEC, which were then washed twice with prewarmed (37°C) PBS, prior to the addition of 5 × 10⁵ PBMC/500 µl pretreated with calcAM to each well. Cells were incubated for 1 h and then were washed very carefully four times with prewarmed PBS. Fluorescence was determined by measurement with the fluorescence reader (Fluoroscan, Labsystems, Helsinki, Finland). Adhesion was automatically expressed in relative fluorescence units. For better comparisons of the differentially treated groups and to avoid the use of relative fluorescence units the adhesion of PBMC pretreated with TNF-α to unstimulated HCEC was chosen as a reference at each incubation time point. The adhesion index is therefore defined as ratio of adhesion of the different treatment groups (in relative fluorescence units) to adhesion of PBMC to unstimulated HCEC (in relative fluorescence units).

For experiments in which the adhesion to HCEC was blocked, cells were treated with monoclonal antibodies to VCAM-1 (Biosource, Camarillo, Calif., USA), ICAM-1 (Bender MedSystems, Vienna, Austria) or isotype mouse IgG1x antibody for 40 min at 37°C/5% CO₂. As an optimal concentration for monoclonal antibodies 2.5 µg/ml was chosen, as determined by previous dilution experiments. HCEC were washed twice with PBS, and PBMC pretreated with calcAM were then added. In addition, PBMC were stimulated for 24 h by treatment with 10 ng/ml TNF-α, washed with PBS and adjusted to 2 × 10⁶ cells/ml, and then incubated with 5, 10 or 100 ng/ml of recombinant soluble VCAM-1 (R&D Systems) or 100 ng/ml monoclonal antibody to VCAM-1 for 40 min at 37°C/5% CO₂ to assess the effect of soluble VCAM-1 on cell adhesion. PBMC were washed twice with RPMI 1640 and then treated with calcAM as outlined above.

**Cytotoxicity assay**

To exclude the possibility of cytotoxic effects of TNF-α and IFN-β treatment in HCEC cultures a cytotoxicity assay was performed. HCEC were stimulated with 0, 1 and 10 ng/ml of recombinant TNF-α alone or in combination with 8 or 80 U/ml IFN-β for 24, 72 and 144 h at 37°C/5% CO₂. Then 250 µl of 100 nM SytoxGreen (Molecular Probes) were added and HCEC incubated for a further 10 min. SytoxGreen
is a fluorescent dye which only penetrates into dead cells. After washing HCEC with PBS, fluorescence was determined using a fluorescence reader.

**Statistical analysis**

All experiments were performed at least in triplicate. Statistical analysis was carried out using the Statview 4.01 software package on an Apple Macintosh computer. Comparison of means was done by Student’s paired t-test. Values of $P < 0.01$ were considered statistically significant.

**Results**

**Cellular VCAM-1 expression and soluble VCAM-1 release in HCEC and PBMC**

Treatment of HCEC with TNF-α induced a dose-dependent upregulation of VCAM-1 expression which peaked after 72 h of incubation and declined at 144 h (Fig. 3A). Simultaneous measurement of soluble VCAM-1 in the cultures revealed a small spontaneous release of soluble VCAM-1 (4 ng/ml) which was enhanced following treatment with TNF-α by threefold at 24 h and fivefold at 72 h. A significant, dose-dependent increase of soluble VCAM-1 release was observed at 144 h in endothelial cells stimulated with TNF-α. Cellular VCAM-1 expression and soluble VCAM-1 release followed different kinetics in the endothelial cell cultures suggesting that soluble VCAM-1 may be released from the cell surface. To exclude the possibility of a toxic effect of TNF-α on endothelial cells with consequent cell destruction and subsequent release of membrane components, cell viability was measured. During the 144 h stimulation period the viability of the endothelial cells remained almost unchanged at 95%. No measurable release of soluble VCAM-1 was observed in the PBMC culture supernatants under the same stimulatory conditions (ELISA detection limit at 1 ng/ml soluble VCAM-1) (data not shown). About 1% of PBMC expressed VCAM-1 spontaneously which increased to 8% at 24 h and 12% at 72 h. No induction of VCAM-1 expression was observed in these cells after stimulation with TNF-α.

We visualized, by immunofluorescence microscopy, the specific expression of VCAM-1 on the surface of HCEC (Fig. 2A–C) which was further increased after stimulation with TNF-α (Fig. 2D–F). In a second series of experiments the HCEC specific VCAM-1 expression was quantitated by double-staining FACS analysis (Fig. 3B). At 72 h, 39.8% of unstimulated HCEC were double stained for *U. europaeus* lectin and VCAM-1. After stimulation with 10 ng/ml TNF-α for 72 h the percentage of VCAM-1 positive HCEC increased to 96.8%.

**Adhesion blockade by monoclonal antibodies to VCAM-1 and ICAM-1**

To investigate the role of VCAM-1 expression in HCEC during cell adhesion we used an adhesion assay in which PBMC, prestimulated with 10 ng/ml TNF-α for 24 h, were coincubated with HCEC under various conditions. To allow comparison of the different experimental conditions at each incubation time point we used an adhesion index. Following stimulation of HCEC with 10 ng/ml TNF-α for 24 h the adhesion index was increased by twofold (Fig. 4). TNF-α-stimulated HCEC pretreated with monoclonal antibody to VCAM-1 (2.5 µg/ml) had a significantly reduced adhesion to PBMC (adhesion index of 1.5). Preincubation of HCEC with monoclonal antibody to ICAM-1 (2.5 µg/ml) blocked the adhesion of PBMC to a lesser extent (adhesion index 1.8). Pretreatment of HCEC with an isotype antibody did not alter the adhesion of PBMC to HCEC when compared with the pure TNF-α-treated endothelial cells in the absence of antibody.

**Adhesion blocked by soluble VCAM-1**

To investigate whether soluble VCAM-1 could interfere with adhesion, PBMC were pretreated with recombinant monomeric soluble VCAM-1 at different concentrations (5, 10, 100 ng/ml) and then added to HCEC (Fig. 5). Adhesion was increased by 2.8-fold when HCEC were stimulated with TNF-α. When PBMC were preincubated with 5 or 10 ng/ml of soluble VCAM-1 the adhesion was reduced by 50%. Preincubation of PBMC with 100 ng/ml soluble VCAM-1 resulted in an almost complete blockade of adhesion (adhesion index 0.2). Incubation of PBMC with a monoclonal antibody to VCAM-1 (100 ng/ml) also reduced adhesion to a level that was similar to that observed when using lower concentrations of soluble VCAM-1 (5 and 10 ng/ml). Adhesion to unstimulated HCEC was blocked to a similar extent by preincubation of PBMC with soluble VCAM-1 at different concentrations (Fig. 5).

**Cellular VCAM-1 expression after treatment with IFN-β**

In contrast to TNF-α, IFN-β (80 U/ml) alone had no effect on VCAM-1 expression after 72 h of incubation (Fig. 6). Furthermore, cotreatment of HCEC with TNF-α and IFN-β did not modulate the cellular VCAM-1 expression induced by TNF-α alone. Similar effects were observed for cellular VCAM-1 expression in HCEC at 144 h (Fig. 6) and were also seen with lower concentrations of TNF-α (1 ng/ml) and IFN-β (8 U/ml) (data not shown).

**Release of soluble VCAM-1 after treatment with IFN-β**

As shown in Fig. 7, a low concentration of soluble VCAM-1 (5 ng/ml) was observed in the supernatant of unstimulated HCEC cultures after 72 h of incubation and these remained almost unchanged after addition of 80 U/ml IFN-β, whereas TNF-α at 10 ng/ml increased the concentration of soluble
VCAM-1 up to 13 ng/ml. The soluble VCAM-1 concentration was further enhanced to 19 ng/ml by coinubcation of HCEC with TNF-α and IFN-β. Significant differences of similar magnitude were found between the various treatment groups after 144 h (Fig. 7). A similar effect on soluble VCAM-1 release in HCEC was also observed at lower concentrations of TNF-α (1 ng/ml) and IFN-β (8 U/ml) (data not shown).

**Discussion**

An increase in soluble VCAM-1 levels has been postulated as an indicator for pathological breakdown of the blood–brain barrier in multiple sclerosis, because it has been shown to positively correlate with relapses (Dore-Duffy et al., 1995) as well as with number and size of gadolinium enhancing lesions on MRI (Hartung et al., 1995; Rieckmann et al., 1997). Recent studies have provided evidence that immunomodulatory treatment with IFN-β also causes an increase of soluble VCAM-1 serum levels in multiple sclerosis patients (Calabresi et al., 1997a; Rieckmann et al., 1998) but the major cellular source of this soluble VCAM-1 has not been identified. Our study demonstrates that soluble VCAM-1 is released from HCEC after stimulation with TNF-α in a time- and dose-dependent manner, while PBMC expressed VCAM-1 at such low levels that no soluble VCAM-1 could be detected in the culture supernatant under identical culture conditions. These results indicate that HCEC may contribute largely to the amount of soluble VCAM-1 in TNF-α-mediated inflammatory diseases of the CNS such as multiple sclerosis as was shown earlier for the release of soluble ICAM-1 (Rieckmann et al., 1995b).

Cellular VCAM-1 expression of and soluble VCAM-1 release from HCEC followed different kinetics. TNF-α treatment initially induced cellular VCAM-1 expression in

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**Fig. 3 (A)** Cellular VCAM-1 expression in HCEC (the percentage of positive cells in total counted cells is indicated in columns) and release of soluble VCAM-1 (in ng/ml, indicated by dots) into the culture supernatant after stimulation with 1 or 10 ng/ml TNF-α. Cultures were incubated for between 24 and 144 h. Results are expressed as means and error bars indicate SD. *P < 0.01 is indicated as **. (B) Flow cytometric analysis of HCEC double-stained with *U. europaeus*-biotin/streptavidin-tricolor and VCAM-1/FITC anti-mouse IgG monoclonal antibodies. Data are shown for HCEC with and without 10 ng/ml TNF-α treatment for 72 h. Percentages of labelled cells are defined by quadrant markers.
HCEC which reached a maximum after 72 h of incubation, while soluble VCAM-1 release increased significantly after 144 h of incubation when the cellular expression had already declined. An increase of soluble VCAM-1 due to a cytotoxic effect of TNF-α was ruled out by measuring cell viability with a fluorescence assay. Therefore, it seems most likely that soluble VCAM-1 is released from the cell surface of HCEC by a shedding mechanism. Initial data from our laboratory indicate that matrix metalloproteases are involved in this process.

Furthermore, the adhesion of PBMC to untreated as well as to TNF-α-pretreated HCEC could be specifically blocked by using monoclonal antibodies to VCAM-1 and ICAM-1. In our culture system VCAM-1 played the major role in adhesion, as blocking VCAM-1 reduced adhesion to a greater extent than blocking ICAM-1 under all conditions studied.
Modulation of cellular adhesion

These results are in accordance with findings of other groups who also showed that VLA-4/VCAM-1 interactions play a major role in adhesion of lymphocytes to endothelial cells at least under inflammatory conditions (Oppenheimer-Marks et al., 1991).

An immunomodulatory role for soluble VCAM-1 has been proposed in rendering T cells hyporesponsive (Kitani et al., 1996) or modulating Th1/Th2 balance. In a previous study we demonstrated that soluble ICAM-1 released from HCEC partly blocked the adhesion of PBMC to HCEC. Here we demonstrated that recombinant monomeric soluble VCAM-1 at concentrations up to 100 ng/ml almost completely blocked adhesion of PBMC to HCEC. Concentrations of soluble VCAM-1 in serum of multiple sclerosis patients during IFN-β treatment ranged from 200 to 900 ng/ml (Rieckmann et al., 1998). Any impact on the cytokine production of PBMC by the interaction of recombinant soluble VCAM-1 with the VLA-4 counter receptor found on PBMC is unlikely to have influenced the adhesion assay because of the short duration of treatment and of the coincubation period of PBMC with HCEC.

In a previous study the effect of IFN-β on endothelial cells was investigated by Dhib-Jalbut and colleagues using HUVEC (human umbilical vein endothelial cells) (Dhib-Jalbut et al., 1996). They found a slight but not significant increase of cellular adhesion molecule expression and of lymphocyte–endothelial cell adhesion after in vitro treatment with IFN-β. Corsini and colleagues also investigated the
modulation of adhesion by IFN-β in HCEC but they used PBMC from multiple sclerosis patients who had received prior treatment with IFN-β (Corsini et al., 1997). In contrast to our in vitro findings, they observed a slight increase of adhesion of PBMC to HCEC.

Having identified HCEC as the major cellular source of soluble VCAM-1 we then investigated whether treatment with IFN-β might further modify the release of soluble VCAM-1. Incubation of HCEC with different concentrations of IFN-β alone did not influence spontaneous release of soluble VCAM-1. However, the release of soluble VCAM-1 induced by treatment of HCEC with TNF-α was significantly increased by coincubation with IFN-β. This effect was found to be time- and dose-dependent. Cellular surface VCAM-1 expression in HCEC after incubation with TNF-α was almost maximal and could not be further modified by cotreatment with IFN-β. In addition, treatment with IFN-β alone did not change VCAM-1 expression in HCEC in comparison with untreated cells.

We therefore propose a sequence of events to describe how disease activity, TNF-α, elevated soluble VCAM-1 serum levels in multiple sclerosis patients and the beneficial effect of IFN-β might be related. Preceding a relapse in multiple sclerosis patients the immunopathological cascade may initially be activated by so far unknown factors stimulating PBMC to produce and release proinflammatory cytokines such as TNF-α, IL1-β or IFN-γ (Chofflon et al., 1992; Rieckmann et al., 1995a). Similarly, activated astrocytes (Sharif et al., 1993) or microglia (Lee et al., 1993) could be the local source of TNF-α at the blood–brain barrier. TNF-α induces the upregulation of adhesion molecules, notably VCAM-1, on HCEC and their counter-receptors on immune cells. This first phase is further characterized by local inflammation and transmigration of immune cells via the blood–brain barrier. In a second phase, as a consequence of TNF-α stimulation, soluble VCAM-1 is released from the endothelial cell surface. Soluble VCAM-1 may then act at different sites, e.g. blocking further adhesion of immune cells to HCEC as evidenced by our study. This consecutive immunomodulatory effect of TNF-α is further enhanced by IFN-β.

Taken together, elevated levels of soluble VCAM-1 in sera of multiple sclerosis patients may reflect two sides of the inflammatory process: on the one hand it may act as an indicator of disease activity during relapses and on the other hand as part of the already initiated feedback downregulation of the inflammatory response which is induced by TNF-α and enhanced by treatment with IFN-β.

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