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# Endothelin-1 enhances vascular cell adhesion molecule-1 expression in tumor necrosis factor α-stimulated vascular endothelial cells

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**Abstract**

Vascular cell adhesion molecule-1 (VCAM-1) is a mononuclear leukocyte-selective adhesion molecule that is expressed in human vascular endothelial cells at sites of local inflammation. It participates in local endothelial–monocyte interactions during the initiation of atherosclerosis. In the present study, endothelin alone did not induce the surface expression and mRNA accumulation of VCAM-1 in human vascular endothelial cells, but inhibition of endogenous nitric oxide (NO) by $N^G$-monomethyl-L-arginine enhanced the surface expression and mRNA accumulation of VCAM-1 stimulated by endothelin-1. It is conceivable that in human vascular endothelial cells, stimulation of an endothelin receptor results in the production of nitric oxide (NO), suppressing the expression of VCAM-1. Endothelin-1 enhanced the surface expression and mRNA accumulation of VCAM-1 in cells treated with tumor necrosis factor α (TNF-α). The enhancement by endothelin-1 may be explained by the inhibitory effect of TNF-α on endothelin-induced NO production. Pretreatment with BQ788 (an endothelin $ET_B$ receptor antagonist) or inhibitors of nuclear factor kappa B (NF-κB) activation completely diminished the synergistic enhancement of VCAM-1 expression by endothelin-1 in TNF-α-stimulated vascular endothelial cells, both at the protein and mRNA levels. These findings suggest that the synergistic enhancement of VCAM-1 expression by TNF-α and endothelin $ET_B$ receptor stimulation may be augmented by the induction of NF-κB binding activity in human vascular endothelial cells. 

*Keywords:* Endothelial cell, human, vascular; Endothelin $ET_B$ receptor; VCAM-1 (vascular cell adhesion molecule-1); NF-κB (nuclear factor kappa B)

## 1. Introduction

Endothelin-1, a 21-amino acid polypeptide with two intramolecular disulphide bounds, is a potent vasoconstrictor peptide synthesized by endothelial cells (Yanagisawa et al., 1988). The three known forms of endothelin (i.e., endothelin-1, -2, and -3) mediate their actions via two distinct receptor subtypes, termed endothelin $ET_A$ and $ET_B$ receptors (Arai et al., 1990; Sakurai et al., 1990). Endothelin-1 and endothelin-2 have equal affinity for the endothelin ETA receptor, while endothelin-3 has much lower affinity: all three forms have equal affinity for the endothelin $ET_B$ receptor (Masaki et al., 1991). Endothelin-1 is strongly expressed under several pathological conditions, e.g., myocardial ischemia (Miyauchi et al., 1989) and atherosclerosis (Lerman et al., 1991), which also involve an interaction between circulating leukocytes and the endothelium.

Vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin superfamily, is a mononuclear leukocyte-selective adhesion molecule expressed in cultured human vascular endothelial cells after activation by inflammatory cytokines such as interleukin-1, tumor necrosis factor α (TNF-α), and interleukin-4 (Bevilacqua et al., 1987; Rice and Bevilacqua, 1989). VCAM-1 has previously been demonstrated in aortic endothelium overlaying atherosclerotic lesions of animal and human arteries (Cybulsky and Gimbrone, 1991; Davies et al., 1993; Li et al., 1993). The serum concentration of circulating VCAM-1 shows significant correlation with the extent of vascular lesions in atherosclerosis (Peter et al., 1997). These studies suggest that the expression of VCAM-1 might be a result of endothelial activation and that VCAM-1 might participate in local endothelial–monocyte interactions during the initiation of atherosclerosis.

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In the vascular wall, endothelin $ET_A$ receptors are mainly localized in vascular smooth muscle cells, whereas endothelin $ET_B$ receptors are found on both vascular smooth muscle and endothelial cells (Namiki et al., 1992). It has been demonstrated that the endothelin $ET_B$ receptor is linked through a guanine nucleotide-binding regulatory protein (Gq protein) to phospholipase C, which hydrolyzes membrane phosphoinositides, resulting in the activation of protein kinase C (Sakurai et al., 1991). A protein kinase C receptor agonist induces nuclear factor kappa B (NF-κB)-like binding activity and the surface expression of VCAM-1 in human vascular endothelial cells (Deisher et al., 1993).

In the present study, it was investigated whether or not endothelin receptor stimulation augments the expression of VCAM-1 in human vascular endothelial cells at the protein and mRNA levels. Inhibitors of NF-κB activation were utilized to determine the intracellular signal pathways by which endothelin-1 stimulates the expression of VCAM-1 in human vascular endothelial cells.

## 2. Materials and methods

### 2.1. Reagents

Recombinant human TNF-α was purchased from Genzyme (Boston, MA, USA). Human endothelin-1, endothelin-2, or endothelin-3 was purchased from Peptide Institute (Osaka, Japan). BQ3020, [$Ala^{11,15}$]Ac-endothelin-$1_{(6-21)}$, an agonist of endothelin $ET_B$ receptors, BQ610, [cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu–], an antagonist of endothelin ETA receptors, and BQ788, *cis*-2,6-dimethylpiperidinocarbonyl-γ-methylleucyl-D-Trp(1-$CO_2CH_3$)-D-norleucyl-OH, an antagonist of endothelin $ET_B$ receptors were provided by Banyu Pharmaceutical (Tsukuba, Japan). 3-Aminobenzamide, an inhibitor of activating protein-1 (AP-1) binding activity, pyrrolidine-dithiocarbamate, an inhibitor of NF-κB activation, and *N*-acetylcysteine, an inhibitor of AP-1 and NF-κB binding activity, were purchased from Sigma (St. Louis, MO). An NO synthase inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA), was purchased from Sigma.

### 2.2. Vascular endothelial cell cultures

Human vascular endothelial cells, collected from human umbilical cord veins by using Jaffe's method (Jaffe et al., 1973), were cultured according to procedures described earlier (Ishizuka et al., 1994). The human vascular endothelial cells used in this experiment were between their 1st and 3rd passages. When cultures reached confluence, the cells were harvested and re-plated at a density of $1 \times 10^6$ cells/dish. The dishes were then washed with a phosphate-buffered saline (PBS). Then, 2 ml of Medium 199 with or without specific inclusions (100 U/ml TNF-α; 20 or 200 nM endothelin-1, endothelin-2, endothelin-3; or BQ3020) was added for 24 h. In some cases, BQ610, BQ788, 3-aminobezamide, pyrrolidine-dithiocarbamate, *N*-acetylcysteine, or L-NMMA was added 15 min prior to the treatment with TNF-α, endothelin-1, endothelin-2, endothelin-3, or BQ3020. The cell number was determined by using a hemocytometer (Erma, Tokyo, Japan). Cell viability was assessed by colorimetric assay with MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrasodium bromide) (Chemicon International, Temecula, CA) (Mosmann, 1983).

### 2.3. Immunofluorescence staining and analysis

Immunofluorescence staining and analysis procedures have been described previously (Ishizuka et al., 1994). Briefly, cells were harvested and washed in PBS containing 1% fetal calf serum. Human γ-globulin (Teijin, Tokyo, Japan) (10 mg/ml) was added to cultures to block nonspecific binding, after which 1 μg/ml of mouse anti-VCAM-1 monoclonal antibody (British Biotechnology, Oxon, UK) was added and the cells were incubated at 4°C for 80 min. After being washed, they were combined with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin G (IgG) antibody (Tago, Burlinggame, CA, USA) and allowed to react at 4°C for 80 min. They were quantitatively analyzed with FACS Calibur™ (Becton Dickinson, San Jose, CA, USA). The percentage of FITC-positive cells was determined as the percentage of cells with a fluorescence intensity that exceeded the threshold obtained with nonspecific mouse IgG, after adjusting the gating scale of the histogram to keep the percentage of positive cells in the negative control below 1%. Mean fluorescence intensity was calculated on a logarithmic scale based on a comparison with a negative control. Results are expressed as the means ± S.D. of three experiments.

### 2.4. Analysis of mRNA in endothelial cells by reverse transcriptase-polymerase chain reaction (RT-PCR)

TNF-α, endothelin-1, endothelin-2, endothelin-3, or BQ3020 was allowed to react with endothelial cell cultures for 4 h, 6 h, or 12 h. Total RNA was isolated using RNA Zol B (Biotecx Labs, Houston, TX). Detection and analysis of gene expression at the RNA level was performed by using a GeneAmp RNA polymerase chain reaction (PCR) kit (Perkin-Elmer Cetus, Norwalk, CT). After a reverse transcription (RT) reaction, using 50 U of Molony murine leukemia virus reverse transcriptase, PCR amplification was performed in a PCR buffer containing 0.15 μM primer A (sense) and B (antisense) and 2.5 U AmpliTaq DNA polymerase in a Perkin-Elmer Cetus thermal cycler. The sequences of the sense and antisense primers for VCAM-1 were 5′-GCTCTGTGACCATGACCTGTTC-3′ and 5′-CTGACCAAGACGTTGTATCTC-3′, respectively.

Amplification of the same RNA with β-actin primers confirmed that equal amounts of RNA were reverse-transcribed. The sequences of the primers for β-actin were 5′-TACATGGCTGGGGTGTTGAA-3′ and 5′-AAGAGAGGCATCCTCACCCT-3′, respectively. The amplification profile was 30 cycles of denaturation at 95°C for 30 s, primer annealing at 56°C for 30 s, and extension at 72°C for 1 min. The PCR products were run on 2% agarose gels (BioRad, Hemel Hempsted, UK) containing 0.5 mg/ml ethidium bromide (Sigma) and compared with DNA reference markers. The gel was visualized under ultraviolet illumination and photographed with a CCD-video camera (AE-6911CX, Atto, Tokyo, Japan). Quantitative image analysis of the PCR fragments on the gel was performed on a Power Macintosh 7500/120 computer (Apple Computer, Cupertino, CA), using a public domain NIH image program (written by Wayne Rashard, National Institutes of Health, Bethesda, MD) (Becker et al., 1996). In the image analysis system, the intensity was calibrated from black (0) to white (255) on a gray scale of 256 channels per pixel. The size and intensity of each band were integrated to quantify the relative amount of PCR products: the relative amount of PCR product (integrated density) = band area × band-specific intensity, where band-specific intensity was calculated as the average intensity per pixel detected in the band area.

### 2.5. Statistical analysis

All results are expressed as the means ± S.D. Changes with respect to basal values when only two observations were made were analyzed by a paired or unpaired Student's *t*-test. Comparisons between the means of multiple groups were analyzed by one-way analysis of variance and Scheffe's multiple comparisons test. Values where $P$ was equal to or less than 0.05 were regarded as significant.

## 3. Results

### 3.1. Effects of endothelin-1, -2, -3, or endothelin $ET_B$ receptor agonist (BQ3020) on the expression of VCAM-1 on the surface of human vascular endothelial cells

The expression of VCAM-1 on the surface of human vascular endothelial cells was 3.5 ± 0.7% without stimulation (mean fluorescence intensity 9.8 ± 0.6) (Table 1). The percentage of VCAM-1-positive cells and mean fluorescence intensity of VCAM-1 was not intensified by the addition with 200 nM endothelin-1, -2, -3, or BQ3020 for 24 h. Endothelin-1 (20 nM or 200 nM), endothelin-2, -3, or BQ3020 (200 nM) was added to human vascular endothelial cells simultaneously with the addition of 100 U/ml of TNF-α (Fig. 1, Table 2). Twenty-four hours after the addition, endothelin-1, -2, -3 or BQ3020 significantly enhanced the expression of VCAM-1 in TNF-α-stimulated vascular endothelial cells ($P < 0.01$; mean fluorescence intensity, $P < 0.01$).

Table 1
Effect of endothelin-1, endothelin-2, endothelin-3, or BQ3020 on the surface expression of VCAM-1 in human vascular endothelial cells

| | Expressing cells (%) | Mean fluorescence intensity |
|---|---|---|
| Nil | 3.5 ± 0.7 | 9.8 ± 0.6 |
| endothelin-1 (200 nM) | 7.4 ± 1.9 | 11.8 ± 1.1 |
| endothelin-2 (200 nM) | 5.0 ± 1.5 | 12.3 ± 5.1 |
| endothelin-3 (200 nM) | 5.7 ± 0.8 | 12.2 ± 2.1 |
| BQ3020 (200 nM) | 5.5 ± 2.0 | 13.9 ± 4.2 |

Human vascular endothelial cells in Medium 199 were exposed to the medium alone (Nil) or incubated with endothelin-1, endothelin-2, endothelin-3, or BQ3020 for 24 h. The cell surface expression of VCAM-1 was analyzed by flow cytometry (as described in Section 2). Results are expressed as the percentage of cells expressing VCAM-1 and mean fluorescence intensity. Data represent the means ± S.D. of three separate experiments.

### 3.2. Effects of endothelin receptor antagonists on the surface expression of VCAM-1 in human vascular endothelial cells stimulated by TNF-α and endothelin-1

To determine whether or not the synergistic enhancement of VCAM-1 expression by TNF-α and endothelins is dependent on the endothelin receptors stimulation in human vascular endothelial cells, the effect of endothelin receptor antagonists on the ability of TNF-α and endothelin-1 to augment VCAM-1 expression on human vascular endothelial cells was evaluated. The doses of BQ610 or BQ788 used in the present study did not affect the cell count or cell viability of human vascular endothelial cells (data not shown). We then assessed the effect of the doses of BQ610 or BQ788 on total protein synthesis. These concentrations of BQ610 or BQ788 did not affect [$^{35}$S]methionine incorporation in human vascular endothelial cells (Table 3).

BQ610 or BQ788 (100 nM, 1 μM, or 10 μM) was added to the human vascular endothelial cells prior to the addition of 100 U/ml of TNF-α or endothelin-1 200 nM. Twenty-four hours after the addition, neither BQ610 nor BQ788 significantly suppressed TNF-α-stimulated VCAM-1 expression (data not shown). The potentiation of VCAM-1 expression of TNF-α and endothelin-1 was dose-dependently suppressed by BQ788, but not by BQ610 (Fig. 2).

### 3.3. Effects of inhibitors of transcription factors on the surface expression of VCAM-1 in human vascular endothelial cells stimulated by TNF-α and endothelin-1

Three agents (3-aminobenzamide, pyrrolidine-dithiocarbamate, and *N*-acetylcysteine) were used to investigate

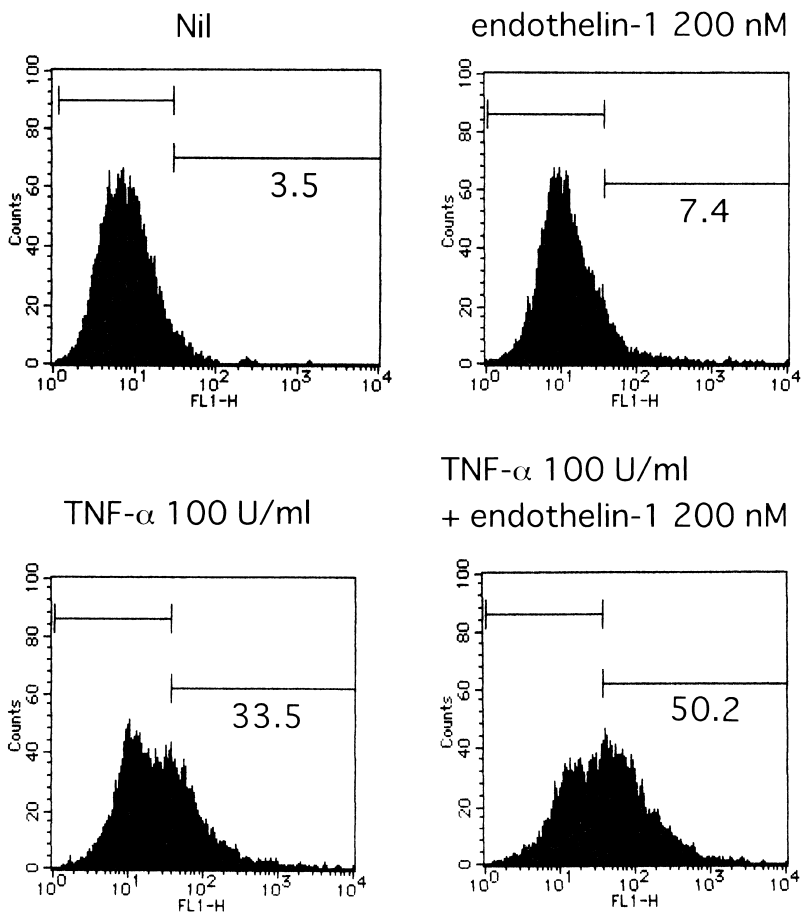


Fig. 1. Plots show effects of TNF-α and endothelin-1 on vascular cell adhesion molecule-1 (VCAM-1) expression in human vascular endothelial cells. Cells were stimulated with endothelin-1 (200 nM) or TNF-α (100 U/ml) for 24 h, stained with specific antibodies for VCAM-1 and analyzed by flow cytometry. Controls consisted of cells incubated with medium alone (Nil). Data indicate the percentage of VCAM-1-expressing cells determined as detailed in Section 2. Shown are original histograms representative of three similar experiments. Relative cell numbers (ordinate) and fluorescence intensity on a $\log_{10}$ scale (abscissa) are indicated.

the possible mechanisms underlying the induction of adhesion molecules. These agents were selected to study the DNA-binding proteins AP-1 and NF-κB, for which the promoter region of adhesion molecules has binding sequences (Voraberger et al., 1991; Neish et al., 1992). An inhibitor of poly-ADP-ribosylation, 3-aminobenzamide, was used to target AP-1 because it inhibits oxidant-induced c-*fos* expression and AP-1-binding activity (Amstad et al., 1992). The antioxidant pyrrolidine-dithiocarbamate, a selective inhibitor of NF-κB activation, was used be-

Table 2
Effect of endothelin-1, endothelin-2, endothelin-3, or BQ3020 on the surface expression of VCAM-1 in TNF-α-stimulated vascular endothelial cells

| | Expressing cells (%) | Mean fluorescence intensity |
|---|---|---|
| Nil | 3.5 ± 0.7 | 9.8 ± 0.6 |
| TNF-α (100 U/ml) | 33.5 ± 3.3 | 26.8 ± 3.4 |
| TNF-α (100 U/ml) + endothelin-1 (20 nM) | 44.3 ± 1.7[a] | 29.9 ± 1.3 |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) | 50.2 ± 3.5[a] | 40.8 ± 1.0[a] |
| TNF-α (100 U/ml) + endothelin-2 (200 nM) | 50.5 ± 1.0[a] | 39.6 ± 1.3[a] |
| TNF-α (100 U/ml) + endothelin-3 (200 nM) | 45.6 ± 0.7[a] | 35.2 ± 1.5[a] |
| TNF-α (100 U/ml) + BQ3020 (200 nM) | 48.1 ± 2.5[a] | 35.8 ± 0.2[a] |

Endothelin-1, endothelin-2, endothelin-3, or BQ3020 was added to monolayers of human vascular endothelial cells upon stimulation with 100 U/ml of TNF-α. Controls consisted of monolayers of human vascular endothelial cells in Medium 199 alone (Nil). The cells were incubated for 24 h. The cell surface expression of VCAM-1 was analyzed by flow cytometry (as described in Section 2). Results are expressed as the percentage of cells expressing VCAM-1 and mean fluorescence intensity. Data represent the means ± S.D. of three separate experiments.
[a] $P < 0.01$ as compared to those stimulated by TNF-α.

Table 3
Effect of BQ610 or BQ788 on protein synthesis in human vascular endothelial cells

| | [$^{35}$S]methionine counts[a] (dpm/μg protein) |
|---|---|
| Nil | 2867.0 ± 156.0 |
| BQ610 (100 nM) | 3618.0 ± 768.0 |
| BQ610 (1 μM) | 3152.0 ± 32.0 |
| BQ610 (10 μM) | 2690.0 ± 106.0 |
| BQ788 (100 nM) | 3761.0 ± 318.0 |
| BQ788 (1 μM) | 3216.0 ± 95.0 |
| BQ788 (10 μM) | 2426.0 ± 241.0 |
| Cycloheximide 10 μg/ml | 349.0 ± 130.0[b] |

[a] Measured as [$^{35}$S]methionine incorporation, as described in Section 2. Monolayers of human vascular endothelial cells in methionine-free medium were incubated with BQ610 (100 nM, 1 μM, or 10 μM), BQ788 (100 nM, 1 μM, or 10 μM), or 10 μg/ml cycloheximide. Controls consisted of monolayers of human vascular endothelial cells in methionine-free medium alone (Nil). The cells were incubated for 24 h. These data are the means ± S.D. of three separate experiments.
[b] $P < 0.01$ as compared to those without stimulation.

cause it acts without affecting the induction of AP-1-binding activity (Schreck et al., 1992). *N*-acetylcysteine, a general anti-oxidant and precursor of glutathione, was used because it inhibits NF-κB- and AP-1-binding activity (Roederer et al., 1990; Bergelson et al., 1994).

Before the addition of TNF-α and endothelin-1, 3-aminobenzamide (10 mM), pyrrolidine-dithiocarbamate (1 mM) or *N*-acetylcysteine (5 mM) was added to human vascular endothelial cells. 3-Aminobenzamide, pyrrolidine-dithiocarbamate, or *N*-acetylcysteine alone did not affect the expression of VCAM-1 (Fig. 3). TNF-α-induced VCAM-1 expression was significantly suppressed by *N*-acetylcysteine ($P < 0.05$), but not by 3-aminobenzamide or pyrrolidine-dithiocarbamate. The potentiation of VCAM-1 expression by TNF-α and endothelin-1 was significantly suppressed by pyrrolidine-dithiocarbamate ($P < 0.01$) and *N*-acetylcysteine ($P < 0.05$), but not by 3-aminobenzamide.

### *3.4. Effects of endothelin-1, endothelin $ET_B$ receptor agonist, antagonists, or inhibitors of transcription factors on the induction of VCAM-1 mRNA accumulation in human vascular endothelial cells*

Endothelin-1 did not induce an increase in the accumulation of VCAM-1 mRNA (Table 4). A subsequent 4-h or 6-h incubation with 200 nM endothelin-1 enhanced the TNF-α-induced accumulation of VCAM-1 mRNA ($P < 0.01$) (Table 4, Fig. 4). TNF-α-induced accumulation of

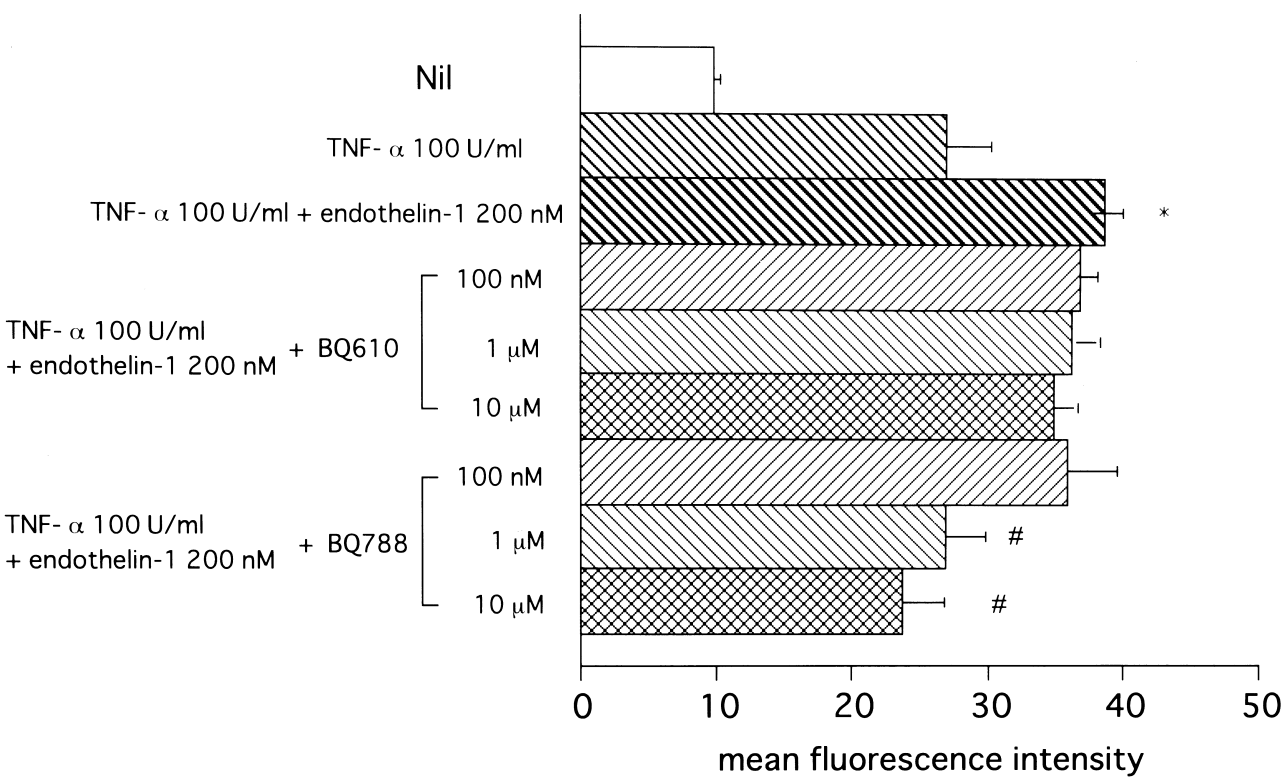


Fig. 2. Effects of BQ610 or BQ788 on the expression of VCAM-1 on the surface of human vascular endothelial cells. BQ610 (100 nM, 1 μM, or 10 μM) or BQ788 (100 nM, 1 μM, or 10 μM) was added to monolayers of confluent human vascular endothelial cells 15 min before the addition of 100 U/ml TNF-α and 200 nM endothelin-1. Twenty-four hours after the addition, the mean fluorescence intensity of the cells was determined as detailed in Section 2. Controls consisted of monolayers of human vascular endothelial cells in Medium 199 alone (Nil). Results are expressed as the means ± S.D. of three experiments. * $P < 0.05$ as compared to those stimulated by TNF-α. # $P < 0.05$ as compared to those stimulated by TNF-α and endothelin-1.

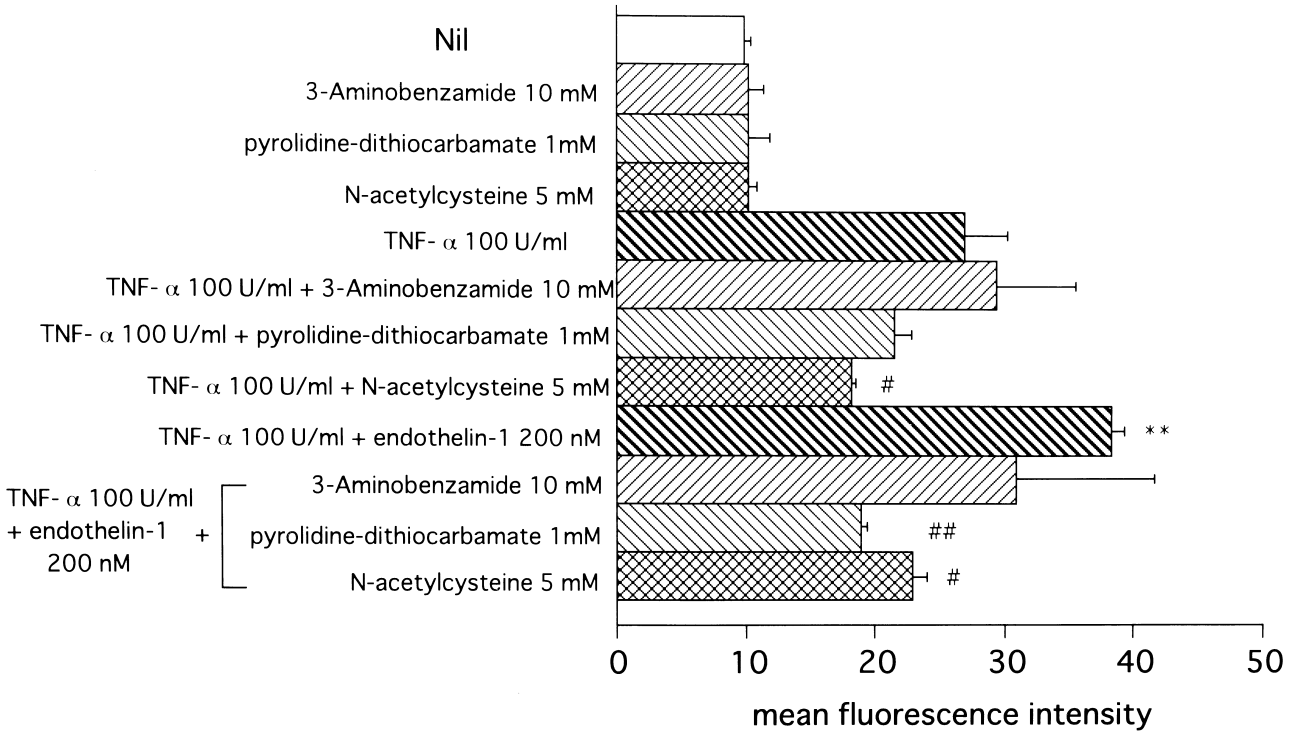


Fig. 3. Effects of 3-aminobenzamide, pyrrolidine-dithiocarbamate, or *N*-acetylcysteine on the expression of VCAM-1 on the surface of human vascular endothelial cells. 3-Aminobenzamide (10 mM), pyrrolidine-dithiocarbamate (1 mM), or *N*-acetylcysteine (5 mM) was added to monolayers of confluent human vascular endothelial cells 15 min before the addition of 100 U/ml TNF-α or 200 nM endothelin-1. Twenty-four hours after the addition, the mean fluorescence intensity of the cells was determined as detailed in Section 2. Controls consisted of monolayers of human vascular endothelial cells in Medium 199 alone (Nil). Results are expressed as the means ± S.D. of three experiments. $^{**}P < 0.01$ as compared to those stimulated by TNF-α. $^{\#}P < 0.05$ as compared to those stimulated by TNF-α or TNF-α + endothelin-1. $^{\#\#}P < 0.01$ as compared to those stimulated by TNF-α or TNF-α + endothelin-1.

VCAM-1 mRNA was enhanced by stimulation with endothelin-2, -3, or BQ3020 (data not shown).

Neither BQ610 nor BQ788 significantly suppressed TNF-α-induced VCAM-1 mRNA accumulation (data not shown). Pretreatment with BQ788 significantly diminished the accumulation of VCAM-1 mRNA in response to TNF-α and endothelin-1 ($P < 0.01$) (Table 4). The VCAM-1 mRNA accumulation induced by TNF-α and endothelin-1 was not reduced by BQ610.

TNF-α-induced VCAM-1 mRNA accumulation was significantly suppressed by pyrrolidine-dithiocarbamate ($25.8 \pm 1.6\% \rightarrow 12.8 \pm 3.6\%$, $P < 0.05$), but not by

Table 4
Effects of endothelin receptor antagonists or inhibitors of transcription factors on the mRNA expression of VCAM-1 in human vascular endothelial cells stimulated by TNF-α and endothelin-1

| | VCAM-1/β-actin mRNA (%) |
|---|---|
| Nil | 4.8 ± 0.5 |
| endothelin-1 (200 nM) | 3.9 ± 0.2 |
| TNF-α (100 U/ml) | 25.8 ± 1.6[a] |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) | 40.5 ± 1.7[b] |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) + BQ610 (10 μM) | 33.8 ± 1.9 |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) + BQ788 (10 μM) | 19.0 ± 0.1[c] |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) + 3-aminobenzamide (10 mM) | 33.4 ± 3.9 |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) + pyrrolidine-dithiocarbamate (1 mM) | 24.0 ± 0.7[c] |

Human vascular endothelial cells were pretreated with or without BQ610, BQ788, 3-aminobenzamide, or pyrrolidine-dithiocarbamate and stimulated by TNF-α (100 U/ml) and endothelin-1 (200 nM). The cells were incubated for 6 h. The reverse transcriptase-polymerase chain reaction (RT-PCR) products were quantified by densitometric analysis as described in Section 2. Data indicate specific PCR products expressed as a percentage in relation to β-actin mRNA (100%) and represent the means ± S.D. of three separate experiments.

[a] $P < 0.01$ as compared to those without stimulation; [b] $P < 0.01$ as compared to those stimulated by TNF-α; [c] $P < 0.01$ as compared to those stimulated by TNF-α and endothelin-1.

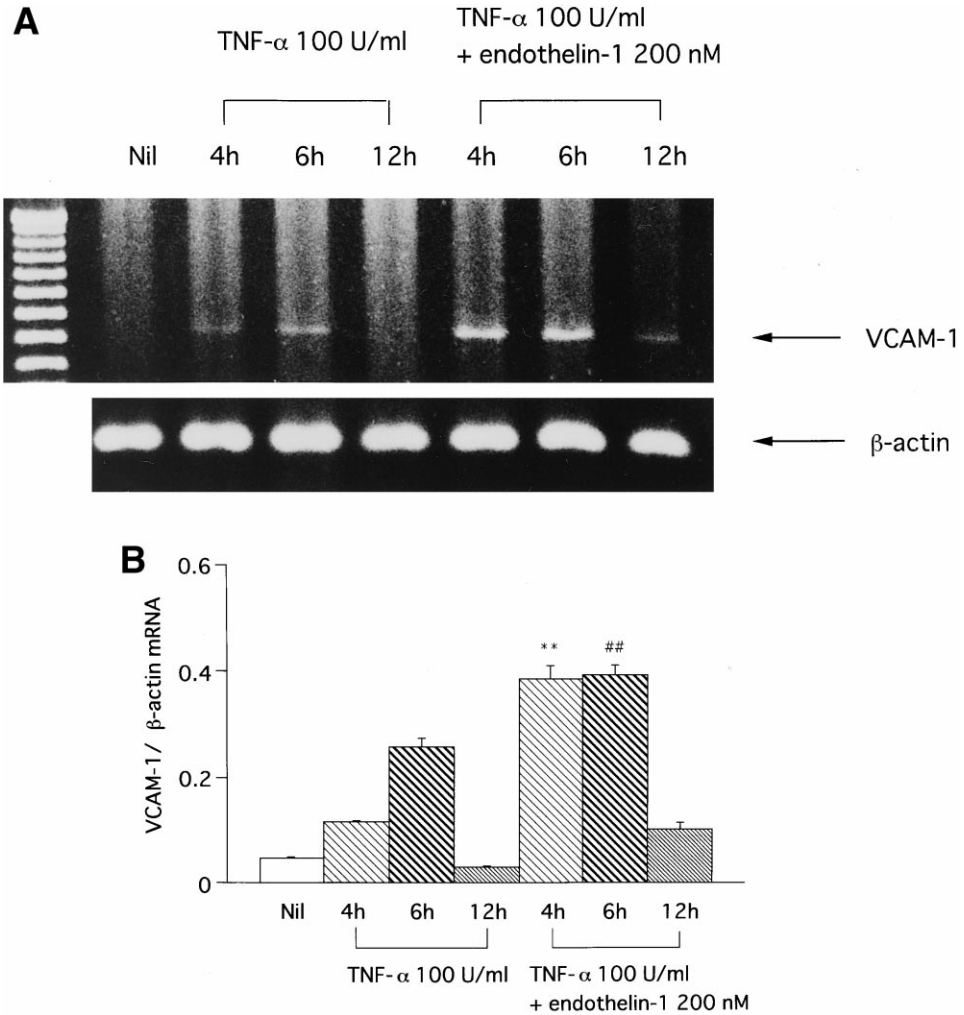


Fig. 4. Effects of endothelin-1 on VCAM-1 mRNA expression in TNF-α-stimulated vascular endothelial cells. Cells were not stimulated, or stimulated with TNF-α (100 U/ml) or TNF-α (100 U/ml) + endothelin-1 (200 nM) and incubated for 4 h, 6 h, or 12 h. Reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted with specific primers for VCAM-1 or β-actin mRNA extracted from $10^7$ cells, as described in Section 2. (A) The PCR products were applied to ethidium-bromide-stained 2% agarose gels and separated by electrophoresis. Shown is a gel photograph representative of three similar experiments. (B) Data indicate specific PCR products expressed as a ratio in relation to β-actin mRNA (1.0) and represent means ± S.D. of three experiments. $^{**}P < 0.01$ as compared to those stimulated by TNF-α for 4 h. $^{\#\#}P < 0.01$ as compared to those stimulated by TNF-α for 6 h.

Table 5
Effect of $N^G$-monomethyl-L-arginine (L-NMMA) on the surface expression and mRNA expression of VCAM-1 in human vascular endothelial cells

| | Surface expression (MFI) | mRNA expression (%) |
|---|---|---|
| Nil | 9.8 ± 0.6 | 4.8 ± 0.5 |
| L-NMMA (10 mM) | 13.2 ± 2.8 | 7.2 ± 2.2 |
| endothelin-1 (200 nM) | 11.8 ± 1.1 | 4.8 ± 0.9 |
| endothelin-1 (200 nM) + L-NMMA (10 mM) | 22.6 ± 0.6[a] | 16.9 ± 0.7[b] |
| TNF-α (100 U/ml) | 26.8 ± 3.4 | 25.8 ± 1.6 |
| TNF-α (100 U/ml) + L-NMMA (10 mM) | 35.0 ± 5.0 | 32.6 ± 3.2 |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) | 40.8 ± 1.0 | 40.5 ± 1.7 |
| TNF-α (100 U/ml) + endothelin-1 (200 nM) + L-NMMA (10 mM) | 35.0 ± 5.0 | 39.0 ± 1.1 |

Human vascular endothelial cells were pretreated with or without L-NMMA (10 mM) and stimulated with TNF-α (100 U/ml) and endothelin-1 (200 nM). The cell surface expression of VCAM-1 was analyzed by flow cytometry. Data are expressed as the mean fluorescence intensity. The expression of VCAM-1 mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Data indicate specific PCR products expressed as a percentage in relation to β-actin mRNA (100%) and represent the means ± S.D. of three separate experiments.
[a] $P < 0.05$ as compared to those stimulated by endothelin-1 200 nM; [b] $P < 0.01$ as compared to those stimulated by endothelin-1 200 nM.

3-aminobenzamide ($25.8 \pm 1.6\% \rightarrow 20.6 \pm 4.0\%$, not significant) (data not shown). VCAM-1 mRNA accumulation induced by TNF-α and endothelin-1 was significantly diminished by pyrrolidine-dithiocarbamate ($P < 0.01$), but not by 3-aminobenzamide (Table 4).

### *3.5. Effects of an NO synthase inhibitor on the surface expression and mRNA accumulation of VCAM-1 in human vascular endothelial cells*

Stimulation of endothelin $ET_B$ receptors results in the release of NO from endothelial cells (Tsukahara et al., 1994). NO can reduce cytokine-induced VCAM-1 expression in human vascular endothelial cells (De Caterina et al., 1995). L-NMMA, a NO synthase inhibitor, was used to determine whether or not NO produced by endothelin-1 stimulation can attenuate VCAM-1 expression in human vascular endothelial cells.

Pretreatment with L-NMMA (10 mM) significantly enhanced the magnitude of the surface expression and mRNA accumulation of VCAM-1 stimulated by endothelin-1 200 nM ($P < 0.05$, $P < 0.01$) (Table 5). In contrast, pretreatment with L-NMMA did not enhance the surface expression and mRNA accumulation of VCAM-1 stimulated by TNF-α and endothelin-1.

## 4. Discussion

The results of the present study show that: (1) stimulation of the endothelin $ET_B$ receptor by endothelin-1 or BQ3020 alone cannot induce the surface expression and mRNA accumulation of VCAM-1 in human vascular endothelial cells (Tables 1 and 4); (2) inhibition of endogenous NO by L-NMMA enhances the magnitude of the surface expression and mRNA accumulation of VCAM-1 stimulated by endothelin-1 (Table 5); (3) stimulation of the endothelin $ET_B$ receptor enhances the surface expression and mRNA accumulation of VCAM-1 induced by TNF-α (Fig. 1, Table 2); (4) pretreatment with BQ788, an endothelin $ET_B$ receptor antagonist, completely diminishes the synergistic enhancement of VCAM-1 expression by endothelin-1 in TNF-α-stimulated vascular endothelial cells at both the protein and mRNA levels (Fig. 2, Table 4). It has been demonstrated that endothelin-1 up-regulates the expression of ICAM-1 or VCAM-1 on fibroblast-like synovial cells. The endothelin-1-induced expression of ICAM-1 or VCAM-1 is mediated via the endothelin ETA receptor (Schwarting et al., 1996). Endothelin ETA receptors mediate endothelin-induced vasoconstriction, whereas endothelin $ET_B$ receptors located on endothelial cells induce a modest vasorelaxation via the release of endothelium-derived NO (Namiki et al., 1992). Endogenous endothelial NO production inhibits VCAM-1 expression in endothelial cell cultures (De Caterina et al., 1995). It is conceivable that, in human vascular endothelial cells, stimulation of endothelin $ET_B$ receptors results in the production of NO, suppressing the expression of VCAM-1. TNF-α down-regulates the expression of endothelium-derived NO synthase through destabilization of endothelial NO synthase mRNA (Alonso et al., 1997). This inhibitory effect of TNF-α on endothelin-1-induced NO production may explain why endothelin-1 can enhance TNF-α-induced VCAM-1 expression at both the protein and mRNA levels.

Endothelin receptor expression in human vascular smooth muscle cells is up-regulated by TNF-α and interleukin-1β (Newman et al., 1995). We considered the possibility that, in human vascular endothelial cells, the expression of endothelin receptors is also up-regulated by TNF-α and that the enhanced binding of endothelin-1 augments the expression of VCAM-1. We feel that further investigations are needed to clarify this point.

Both human endothelin ETA and $ET_B$ receptors can couple with a pertussis-toxin-insensitive Gq protein to activate phospholipase C (Takuwa et al., 1989). Activation of phospholipase C leads to the breakdown of phosphatidylinositol 4,5-bisphosphate, resulting in the formation of inositol 1,4,5-triphosphate and diacylglycerol. These release intracellular $Ca^{2+}$ and stimulate protein kinase C (Berridge, 1987). The surface expression of VCAM-1 was induced by agonists of protein kinase C in human vascular endothelial cells. Protein kinase C agonists induced NF-kB-like binding activity, which has been shown to be necessary for the expression of VCAM-1 (Deisher et al., 1993). The promoter region of the VCAM-1 gene has consensus binding sequences for NF-kB, and deletion or mutation of these NF-kB binding sequences in the VCAM-1 promoter prevents transcription. Pretreatment with inhibitors of NF-kB activation completely diminished the synergistic enhancement of VCAM-1 expression by endothelin-1 in TNF-α-stimulated vascular endothelial cells at both the protein and mRNA levels (Fig. 3, Table 4). These findings suggest that the synergistic enhancement of VCAM-1 expression in human vascular endothelial cells stimulated via endothelin $ET_B$ receptors may be augmented by the induction of NF-kB binding activity.

Oxidized low-density lipoproteins in atherosclerotic blood vessels can stimulate endothelin production in the human endothelium (Boulanger et al., 1992). An increase in the endothelin plasma levels occur in atherosclerosis, myocardial infarction, and arterial hypertension (Lerman et al., 1991; Stewart et al., 1991; Luscher et al., 1993). It has been assumed that TNF-α is not expressed in normal arteries, whereas it is up-regulated in atherosclerosis (Ross, 1993). An elevated level of TNF-α expression is associated with the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbits (Lei and Buja, 1996). Therefore the results shown in this study suggest that the synergistic effect on VCAM-1 expression of TNF-α and endothelins may alter the adhesive properties of the endothelial lining and thereby promote the process of atherosclerosis.

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