CYTOKINE-INDUCED ADHESION MOLECULE EXPRESSION ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS IS NOT REGULATED BY CYCLIC ADENOSINE MONOPHOSPHATE ACCUMULATION

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Summary

We examined the effect of agents which augment intracellular levels of cyclic adenosine monophosphate on the expression of adhesion molecules on human umbilical vein endothelial cells. Surface protein expression of vascular cell adhesion molecule-1, endothelial leukocyte adhesion molecule-1, or intercellular adhesion molecule-1, which is induced by tumor necrosis factor, interleukin-1, and lipopolysaccharide, was not induced by pentoxyfilline, a phosphodiesterase inhibitor, nor by dibutyryl cyclic adenosine monophosphate. Furthermore, neither of these two cyclic adenosine monophosphate elevating agents nor HA 1004, an inhibitor of the cyclic adenosine monophosphate-dependent protein kinase, had any effect on tumor necrosis factor-α-induced surface expression of these adhesion molecules. Likewise, cyclic adenosine monophosphate elevating agents were without effect on leukocyte adherence to endothelium stimulated either with these agents alone or in combination with tumor necrosis factor-α. Additionally, activators of the stimulatory or inhibitory guanine nucleotide-dependent binding proteins did not affect TNF-α-induced surface expression of endothelial leukocyte adhesion molecule-1 or vascular cell adhesion molecule-1.

Tumor necrosis factor (TNF), interleukin-1β (IL-1β), lipopolysaccharide (LPS) and protein kinase C agonists induce the surface expression of the adhesion molecules endothelial leukocyte adhesion molecule-1 (ELAM-1), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) in human umbilical vein endothelial cells (HUVE) (for review see 1). The intracellular signalling pathways utilized by TNF, IL-1β, and LPS remain elusive. A role for cyclic adenosine monophosphate (cAMP) as a second messenger in HUVE signalling pathways has been suggested by several studies. For instance, cAMP and pentoxyfilline have been found to enhance bovine pulmonary artery barrier function (2,3), and cAMP has also been reported to be an important determinant of human dermal microvascular endothelial cell permeability (4). Also, Langeler and van Hinsburgh (5) found that norepinephrine and iloprost, both cAMP elevating agents, increased transendothelial resistance in human umbilical artery endothelial cells.

The possibility that cAMP functions as a second messenger to induce adhesion molecule expression has been raised by two studies. Firstly, Turunen et al. (6) reported that forskolin, IL-1 and dibutyryl cyclic adenosine monophosphate (dBcAMP) treated rat heart endothelial cells had greater lymphocyte penetration, and showed that IL-1 increased cAMP levels, with a peak increase at 15 minutes. The effect of IL-1 was reduced by dideoxyadenosine, an inhibitor of adenylyl cyclase. And secondly, Sung et al. (7) recently reported that cholera toxin, forskolin, dBcAMP, isoproterenol, epinephrine, and norepinephrine increase the adhesion of U937 cells to stimulated HUVE, and that this increased adhesion was directly correlated with an increase in cAMP levels. Also, Imamura et al. (8) have found evidence for an interaction between TNF, the TNF receptor and GTP-dependent binding proteins in HL-60 cells.

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However, work by Pober (9) questioned the role that cyclic adenosine monophosphate may play in inducing adhesion molecule expression. Pober found that neither cAMP nor cGMP induced binding of the antibody H4/18, which recognizes E-selectin (ELAM-1), to HUVE.

Since TNF and IL-1 have been shown to increase cAMP levels in human fibroblasts (10,11), and since the monocytic cell line, U937, used in the study by Sung et al (7), adheres to endothelium via the adhesion molecules ELAM-1 and VCAM-1 (12,13), and since previous studies have not resolved the question of cyclic adenosine monophosphate as a second messenger for cytokine stimulation of HUVE, we evaluated the ability of cyclic adenosine monophosphate elevating agents to alter adhesion molecule expression on HUVE. We also evaluated the effect of these agents on resting leukocyte adherence to activated endothelium.

Our studies question the role of cyclic adenosine monophosphate as a second messenger for TNF, IL-1 or LPS in HUVE. Furthermore, we found no evidence that cyclic adenosine monophosphate, by itself, affects adhesion molecule expression, nor does TNF appear to stimulate HUVE via interaction with a GTP-binding protein.

Materials and Methods

Culture of Endothelial Cells

Human umbilical vein endothelial cells were obtained by collagenase treatment of human umbilical veins (14), and cultured in RPMI 1640 medium (Whittaker Bioproducts Inc., Walkersville, MD) supplemented with 10% adult bovine serum (Hyclone Sterile Systems, Logan, UT), 10% normal calf serum (Armour Pharmaceutical Co., Kanakee, IL), 90 μg/ml heparin (Sigma Chemical Co., St. Louis, MO), and 50 μg/ml endothelial cell growth factor (15), which was prepared from bovine hypothalamus as described by Maciag et al. (16). Cells were used from passage one to three in all experiments.

ELISA

Human umbilical vein endothelial cells were plated in 96 well plates (Costar, Data Packaging Corp., Cambridge, MA) and used at confluency as determined by inversion microscopy. Unless otherwise specified, cAMP-elevating agents were added to the plates 15 minutes prior to the addition of cytokines. After a 5.5 hour incubation in the presence of the cytokines the plates were inverted and washed twice with RPMI 1640 medium supplemented with 2% newborn calf serum (RPMI 1640/2%NCS) (Gibco Laboratories, Long Island, NY), prior to the addition of the anti-ELAM-1 monoclonal antibody (mAb) BB11 (17), the anti-VCAM-1 mAb 4B9 (18), or the anti-ICAM-1 monoclonal antibodies LB2 or R6.5. After a one hour incubation with the first antibody the plates were washed twice with RPMI 1640/2%NCS, and fixed with 0.05% glutaraldehyde for 10 minutes at room temperature. The plates were then washed with RPMI 1640/2%NCS, and a peroxidase-labeled goat anti-mouse second antibody (Tago Laboratories, Bundoora, CA.) was applied for a second one hour incubation at 37°C. The plates were then washed with PBS, 1mg/ml o-phenylenediamine-peroxide was added, and absorbance read at 490nm on a Titertek Multiscan MCC/340 (Flow Lab., McLean, VA).

Adherence Assay

U937 cells were labelled with 51Cr, as previously described (19). Human umbilical vein endothelial cells were plated onto gelatin-coated 48 well tissue culture plates, and allowed to reach confluence. Tumor necrosis factor, PMA, or cAMP elevating agents were incubated with the HUVE for 4 hours at 37°C. Labeled U937 cells were then added to the wells (500,000 cells/well), and the plate was incubated at 37°C for 30 minutes. The supernatant was then decanted, and the wells were then washed twice with PBS(-). Adherent cells were lysed with 0.5ml of 1N NH4OH. Percent adherence was calculated by the formula

\[
\% \text{Adherence} = \left( \frac{51\text{Cr cpm in lysate}}{\text{Total 51Cr cpm added}} \right) \times 100
\]

Reagents

Tumor necrosis factor-α and mAb BB11 were gifts of R. Lobb (Biogen Inc., Cambridge, MA), mAb LB2 was a gift of E. Clark (University of Washington, Seattle, WA.), and mAb R6.5 was a gift of R. Rothlein (Boehringer Ingelheim, Ridgefield, CN.). Interleukin-1β was purchased from R & D Systems Inc. (Minneapolis, MN), and lipopolysaccharide was purchased from List Biologicals Inc. (Campbell, CA). All other reagents were purchased from standard commercial sources.
Results

ELISA

Effect of cAMP-elevating agents or cAMP-dependent protein kinase inhibition

The cAMP-dependent protein kinase inhibitor, HA1004 (25μM up to 200μM) did not prevent TNF-α-, IL-1β-, or LPS- induced surface expression of ELAM-1 or VCAM-1 (the results with TNF-α are depicted in Figure 1, other data not shown). Similarly dBcAMP did not induce their expression (10^{-6}M up to 10^{-4}M), nor did it influence TNF-α-induced surface expression of ELAM-1 or VCAM-1 (Figure 1). Dibutylate cAMP also did not induce the surface expression of ICAM-1 (Figure 2), nor did it affect PMA-induced surface expression of ELAM-1 or VCAM-1 (data not shown). The phosphodiesterase inhibitor, pentoxyfilline, at concentrations from 500nM up to 10mM, likewise had no affect on the surface expression of ELAM-1 or VCAM-1 by itself, or in combination with TNF-α, IL-1β, or LPS (the results with TNF-α are depicted in Figure 1, other data not shown).

The effect of pentoxyfilline, dBcAMP and HA1004 on the surface expression of ELAM-1 and VCAM-1.

Human umbilical vein endothelial cells were treated with pentoxyfilline, dBcAMP or HA1004 for 15 minutes prior to the addition of TNF-α (10ng/ml). After a further 5.5 hour incubation with or without TNF-α, ELAM-1 and VCAM-1 surface expression were measured by ELISA as described in Methods. One representative experiment, out of four total, is depicted for each agent at one or more of the concentrations tested.
FIG. 2.

TNF-α- or dBcAMP-induced surface expression of ELAM-1, VCAM-1, and ICAM-1 on HUVE. Concentration-response curves for TNF-α and dBcAMP induction of ELAM-1, VCAM-1 and ICAM-1 surface expression were generated. TNF-α induced ELAM-1, VCAM-1, and ICAM-1 surface expression on HUVE in a concentration-dependent manner, while dBcAMP did not induce the surface expression of these adhesion molecules.

Effect of GTP binding proteins and adenylate cyclase inhibition

Dideoxyadenosine (1mM), an inhibitor of adenylate cyclase, had no effect on TNF-α-induced ELAM-1 or VCAM-1 surface expression. GTPγS and NaF, activators of the stimulatory GTP-dependent binding protein, also had no effect on TNF-α-induced ELAM-1 or VCAM-1 surface expression. GTPγS was used at concentrations from 250μM up to 1mM, and NaF was used at concentrations from 50μM up to 10μM. An 18 hour pretreatment with pertussis toxin (10ng/ml), an activator of the inhibitory GTP-binding protein, or with cholera toxin (10ng/ml), an activator of the stimulatory GTP-binding protein, likewise, did not influence TNF-α-induced surface expression of ELAM-1 or VCAM-1 (data not shown).

Adherence Assay

Resting Leukocyte Adherence to Activated Endothelium

The effect of agents which result in the intracellular accumulation of cAMP on the adherence of resting leukocytes to endothelium was also investigated in four experiments. Isoproterenol (5-20μM), dBcAMP (1-4μM), forskolin (5-20μM), cholera toxin (250ng/ml), or IBMX (250-500μM) did not affect the adherence of unstimulated U937 cells to HUVE treated with these agents alone or in combination with TNF-α (10ng/ml) (Figure 3).
FIG. 3.

Adherence of resting leukocytes to HUVE stimulated with dBcAMP, forskolin, or isoproterenol in the presence or absence of TNF-α.

A. Human umbilical vein endothelial cells were treated with dBcAMP, forskolin, isoproterenol or TNF-α (10ng/ml) for 4 hours at 37°C in the presence or absence of 0.5mM IBMX (n=1). B. Human umbilical vein endothelial cells were treated with dBcAMP, forskolin, or isoproterenol in the presence of TNF-α (10ng/ml) and the presence or absence of 0.5mM IBMX for 4 hours at 37°C. U937 cells, labeled with 51Cr, were then incubated with the HUVE monolayer for 30 minutes at 37°C, and adherence measured as described in Methods (n=2). Cyclic AMP accumulation did not alter the adherence of resting leukocytes to TNF-α-stimulated endothelium.

Discussion

The results of this present study indicate that cAMP does not function as an intracellular second messenger for TNF-α, II-1B, or LPS induction of adhesion molecule surface expression on HUVE. Agents that stimulate the accumulation of intracellular cAMP did not induce the surface expression of ELAM-1, VCAM-1, or ICAM-1. Likewise, these agents did not affect the surface expression of ELAM-1 or VCAM-1 induced by TNF-α, II-1B, or LPS. Furthermore, adhesion of resting leukocytes to activated endothelium was also unaffected by these agents. In addition, guanine nucleotide-dependent binding proteins do not appear to interact with TNF and its receptor on HUVE.

Our results are in agreement with those of Pober (8), who found that neither cAMP nor cGMP induce the surface expression of ELAM-1 on HUVE. In contrast, Turunen et al. (6) and Sung et al. (7) observed an effect of cAMP on endothelial permeability to lymphocytes and endothelial adhesiveness for U937 cells, respectively. Turunen et al. (6) were working with rat heart endothelial cells, and this species difference may account for the discrepancies. However, Sung et al. (7) also studied HUVE. The reasons for the differences in our results are uncertain.

Although cyclic adenosine monophosphate has been shown to regulate the adherence of leukocytes to the endothelium (7,20,21), our results suggest that this effect is probably mediated via accumulation of cAMP in the adherent cells (20,21), and not a direct effect of cAMP on adhesion molecule expression on HUVE.
References