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Expression of Vascular Cell Adhesion Molecule-1 on Human Pulmonary Artery Endothelial Cells and Human Umbilical Vein Endothelial Cells Stimulated by Tumor Necrosis Factor-α, Interleukin-1β and Lipopolysaccharide

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It has been recently suggested that there is a diversity of endothelial cells (ECs) from different organs in response to cytokines and lipopolysaccharide (LPS). We examined whether there was a differential response among human pulmonary artery ECs (HPAECs) and human umbilical vein ECs (HUVECs) with regard to the expression of vascular cell adhesion molecule-1 (VCAM-1) by tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and LPS stimulation. VCAM-1 expression on these ECs stimulated by the stimulators was measured by cellular enzyme-linked immunosorbent assay (ELISA) and flow cytometric analysis. For confirmation of our use of cells as ECs, the expression of Factor VIII and CD36 were also measured by flow cytometric analysis. Factor VIII was expressed on unstimulated HPAECs and HUVECs at positive cell, 43.1% and 28.9%, respectively, but not CD36 on these ECs entirely. VCAM-1 was not expressed on unstimulated HPAECs and HUVECs. Maximal expression of VCAM-1 on these ECs was induced 6 h after stimulation by TNF-α, IL-1β and LPS at doses of 10 ng/mL, 1 ng/mL and 1 μg/mL, respectively. There was a significant difference in the maximal expression between these ECs ($P < 0.05$; Mann-Whitney U test). Furthermore, VCAM-1 expression by any stimulation was invariably greater on HPAECs than on HUVECs throughout the course of the experiment ($P < 0.01$; two-way ANOVA). Therefore, we suggested that ECs from different organs might have diversity with regard to VCAM-1 expression by TNF-α, IL-1β and LPS stimulation.

Key words: cellular ELISA; endothelial cells; VCAM-1

Endothelial cells (ECs) play key roles in the creation of the barrier to endotoxin and interaction with inflammatory cells during migration into the specific organs. Susceptibility of cultured ECs to endotoxin-induced injury varies among species and among sites of origin of the cells. The pulmonary artery cell line (Harlan et al., 1983a) and kidney microvascular cell line (Raghu et al., 1986) are quite susceptible to the effects of endotoxin whereas human umbilical vein ECs (HUVECs) (Harlan et al., 1983b; Raghu et al., 1986) and human omentum (Smedly et al., 1986) are particularly resistant.

The interaction between ECs and inflammatory cells is now considered to form an important part of the inflammatory process through some adhesion molecules. ECs are stimulated by proinflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), as well as endotoxin, to increase expression of cell surface adhesion molecules, leading to dramatically altered interactions with leuko-

Abbreviations: ANOVA, analysis of variance; EC, endothelial cell; ELAM-1, endothelial-leukocyte adhesion molecule-1; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell scan; FITC, fluorescein isothiocyanate; HPAEC, human pulmonary artery EC; HUVEC, human umbilical vein EC; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukin-1β; LPS, lipopolysaccharide; mAb, monoclonal antibody; MFI, mean fluorescence intensity; OD, optic density; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1

cytes, e.g., granulocytes and monocytes. In these interactions, endothelial-leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are known to play an important role, as they are presented by the ECs and interact with corresponding ligands on the leukocyte membranes (Carlos and Harlan, 1994). VCAM-1 is an especially important adhesion molecule that is induced on ECs by cytokine- and lipopolysaccharide (LPS)-stimulation, and can mediate the binding of lymphocytes, eosinophils or monocytes to the ECs in inflammatory lung diseases, e.g., bronchial asthma (Ohkawara and Yamauchi, 1993) and adult respiratory distress syndrome (Grau et al., 1996).

Until now, the regulation of these proteins, including ELAM-1, ICAM-1 and VCAM-1, has been most often studied on ECs derived from the umbilical vein (Deisher et al., 1993; Gille et al., 1996; Grau et al., 1992; Swerlick et al., 1992b). It is suspected that the effect of VCAM-1 expression by stimulation of ECs are diverse and complicated, and vary between species and in cellular origin. Swerlick and colleagues (1992a) have already reported that there was a significant difference in VCAM-1 expression between human dermal ECs and HUVECs.

We considered that it was necessary to examine the regulation of expression of VCAM-1 on human pulmonary artery ECs (HPAECs) in airway inflammation models. The aim of this study was to compare the reaction profiles of VCAM-1 expression on HPAECs and on HUVECs obtained from the same passages after defined periods of exposure to cytokines and LPS.

Materials and Methods

Endothelial cell monolayers

HPAECs and HUVECs were purchased from Kurabo Co. Ltd. (Osaka, Japan). HPAECs came from a single donor and were received cryopreserved (5×10^5 cells) in the 3rd passage. HUVECs were received in the 1st passage. The cells were cultured separately at 37°C in 5% CO_2 by using EC growth medium consisting of modified Hu-Media EB (Kurabo) supplemented with 10 ng/mL recombinant endothelial growth factor, 5 ng/mL recombinant fibroblast growth factor, 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B sulfate and 2% fetal bovine serum in culture dishes (Falcon 3002, Becton Dickinson Labware, Lincoln Park, NJ). Experiments were carried out using cells at the 5th passage, and HUVECs or HPAECs were used for experiments when they had formed confluent monolayers in culture dishes. The confluent ECs were removed from culture dishes with 0.125% EDTA (Kurabo) in phosphate buffered saline (PBS) (Research Institute for Medical Disease, Osaka), centrifuged and resuspended in EC growth medium at a concentration of 2×10^4 cells/mL. The ECs were then cultured overnight on 0.1% gelatin (Sigma, St. Luois, MO)-coated 96-well microtiter plates (Nunc, Roskilde, Denmark). The ECs were stimulated by TNF-α, IL-1β and LPS at the desired final concentration. After stimulation for the required length of time, the wells were washed with warm EC growth medium. The cells were confirmed to be ECs by morphologic criteria and flow cytometric analysis.

Monoclonal antibodies and stimulator

The monoclonal antibody (mAb) directed against VCAM-1 (CD106; I.G 11B1) and the von Willebrand factor (Factor VIII) [fluorescein isothiocyanate (FITC)-conjugated] were purchased from Serotec Inc. (Bicester, United Kingdom). The anti-CD36 mAb (IgG1, FITC-conjugated) was purchased from Immunotech Co. (Marseille, France). The biotinylate goat anti-mouse Ig [$(Fab)^2$ fragment] was purchased from Jackson ImmunoResearch Lab. (Avondale, PA). The phycoerythrin-conjugated antibody was purchased from Organon Teknika (Durham, NC). Recombinant human TNF-α and IL-1β were purchased from Genzyme Diagnostics (Boston, MA). LPS (*E. coli*, 055, B5) was purchased from Difco Laboratories (Detroit, MI).

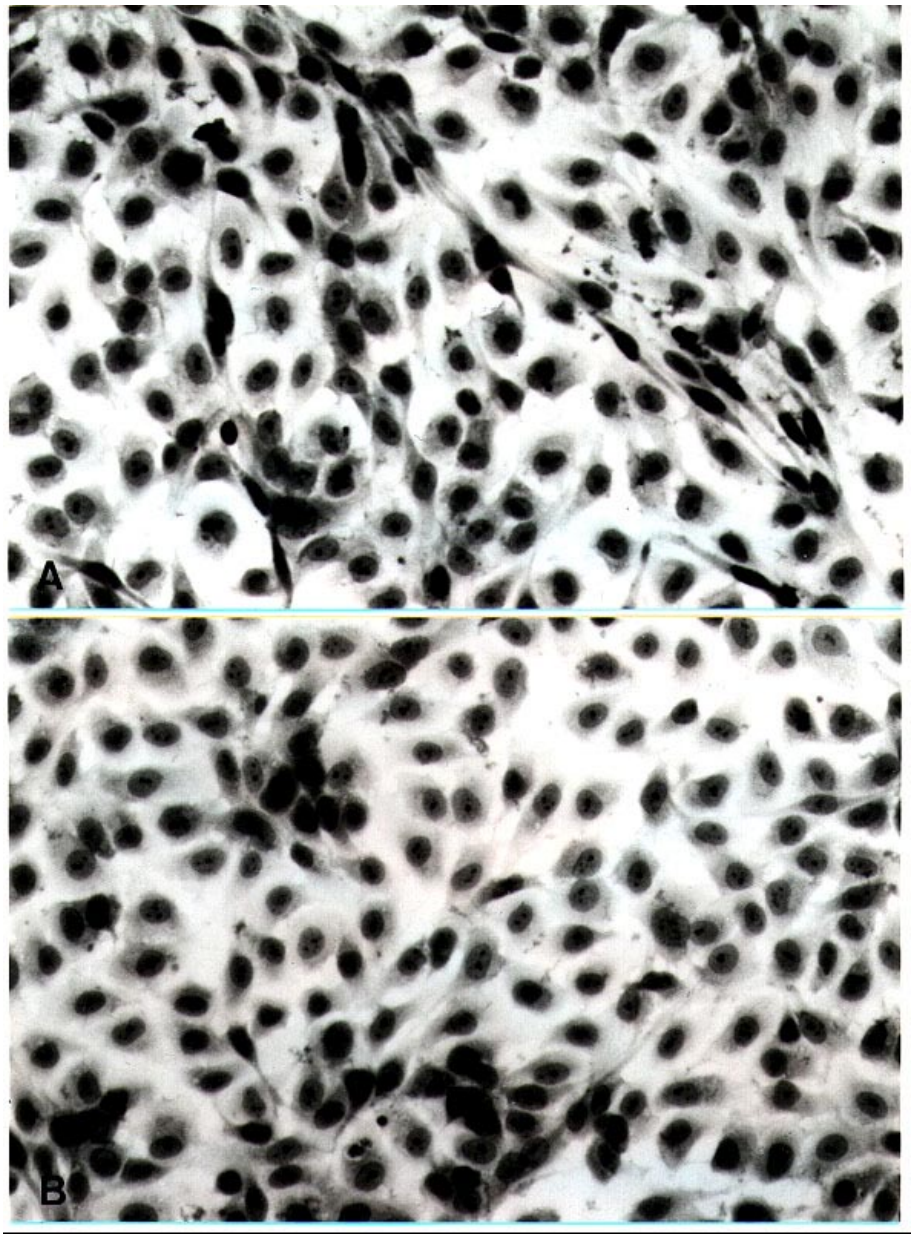

Fig. 1. Micrographs of May-Grünwald Giemsa staining of human pulmonary artery endothelial cells (HPAECs) (**A**) and human umbilical vein endothelial cells (HUVECs) (**B**) of the 5th passage cells. **A** and **B**, × 200.

Cellular enzyme-linked immunosorbent assay

The EC monolayers were fixed by incubating with a solution of 2% paraformaldehyde, 0.075% L-lysine monohydrochloride and 2.1 mg/mL solution m-periodate for 10 min. This procedure has been shown to preserve the antigenicity of many protein and carbohydrate determinations (Van Ewijk et al., 1980; Van Duinen et al.,

1984). Fixation was stopped by aspiration of a fixative, washing the cells with 100 mM glycine, 0.1% bovine serum albumin (BSA) (Sigma) in Hank's balanced salt solution (Bio Whittaker,Walkersville, MD) (blocking solution). The assay plates were stored at 4°C with 200 µL/well blocking solution. An enzyme-linked immunosorbent assay (ELISA) was used, as previously described (Cui et al., 1983), to measure the expression of VCAM-1 on ECs. The ELISA was performed at room temperature after 3 times washing with 0.1% BSA in PBS. The ECs were incubated for 1 h with anti-VCAM-1 antibody (CD106). After 3 times washing with PBS, biotinylated goat anti-mouse Ig was added. After 3 times washing with PBS, the enzyme substrate 0.5 mg/mL o-phenylenediamine and 0.03% hydrogen peroxide in citrate-phosphate buffer (Sumitomo Bakelight Co., Tokyo, Japan) was then added. Color development was stopped with 2 N sulfuric acid, and the optic density (OD) of each well was read at 492 nm in a Micro plate reader (Tosoh Ltd., Tokyo). Test and control measurements were performed 5 times in each experiment, and the degree of specific mAb binding was calculated by subtracting the mean negative control value from each test value. The results were expressed as mean ± SD for each set of measurements. Negative control values differed from experiment to experiment but were always less than 0.01 at OD.

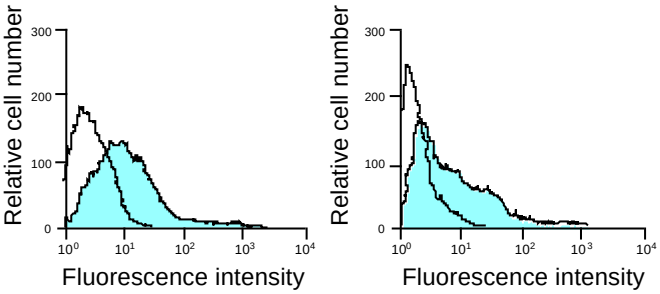

Fig. 2. Flow cytometric analysis of von Willebrand factor on HPAECs (**A**) and HUVECs (**B**). HPAECs and HUVECs were stained by anti-von Willebrand factor monoclonal antibody and were analyzed by a flow cytometry as described in Materials and Methods. Von Willebrand factor was expressed 30 to 40% on HPAECs and HUVECs.

Immunofluorescence staining and flow cytometry

The expression of surface antigens on HPAECs and HUVECs was detected by the binding of monoclonal antibodies to VCAM-1 (CD 106), CD36 and von Willebrand factor (Factor VIII) for 90 min on ice. After washing the cells 3 times with PBS containing 0.1% sodium azide (Wako, Osaka), the expression of CD36 and Factor VIII was evaluated by indirect fluorescence after incubation with either FITC- or phycoerythrin-conjugated antibodies (Organon Teknika) for 30 min on ice in the dark. VCAM-1 expression was evaluated by direct immunofluorescence staining with FITC-conjugated primary antibody. Flow cytometry was performed with 10,000 cells per sample on a fluorescence activated cell scan (FACS) analyzer (Becton Dickinson, Mountain View, CA). The ECs were analyzed by selective gating based on forward and side scatter parameters. Results were expressed as mean fluorescence intensity (MFI), which reflects the cells surface density of the respective marker, and were compared with those obtained with unlabelled cell and isotype controls

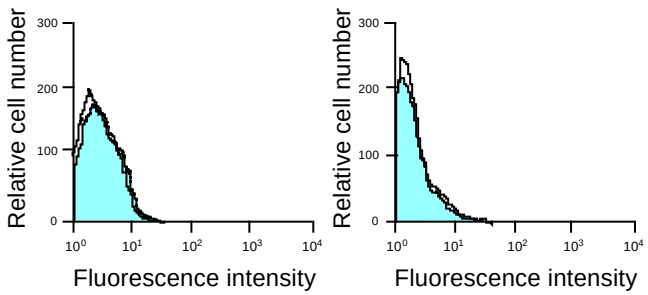

Fig. 3. Expression of CD36 on HPAECs (**A**) and HUVECs (**B**). Unstimulated HPAECs and HUVECs were stained by anti-CD36 monoclonal antibody and were analyzed by flow cytometry as described in Materials and Methods. CD36 was not expressed on HPAECs and HUVECs.

(Becton Dickinson) with the use of the Lysis II program (Becton Dickinson).

Statistical analysis

Data were presented as mean ± SD. The statistical analysis of difference between HPAECs and HUVECs was performed by two-way ANOVA and the nonparametric Mann-Whitney U unpaired test. *P* values less than 0.05 were considered to be significant. These statistical analyses were performed with the Stat View 4.11 statistics package (Abacus Concepts, Berkeley, CA).

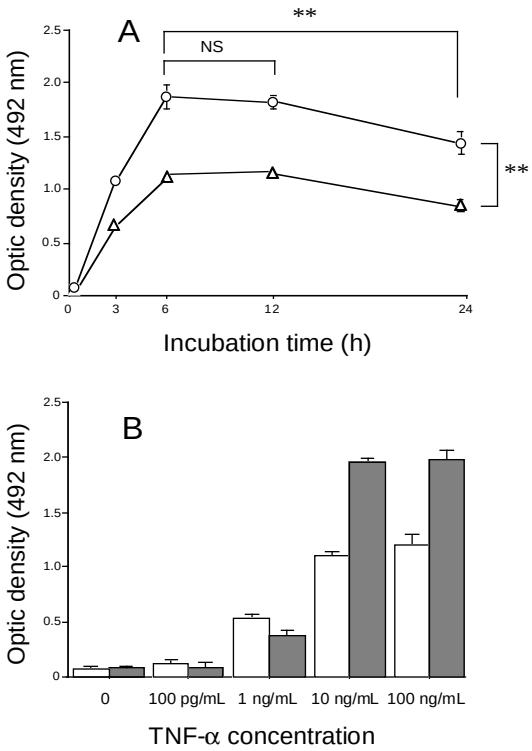

Fig. 4. VCAM-1 expression on HPAECs and HUVECs measured by cellular ELISA after TNF-α stimulation. HPAECs (o) and HUVECs (Δ) in the time-dependent manner were stimulated by TNF-α (10 ng/mL) (**A**). HPAECs (hatched columns) and HUVECs (open columns) in the dose-dependent manner were stimulated by TNF-α for 6 h (**B**). Values are mean ± SD; $n = 5$. Significantly different (**$P < 0.01$) by analysis with two-way ANOVA and the nonparametric Mann-Whitney *U* test between HPAECs and HUVECs in the time-dependent manner. NS, not significant.

Results

Morphology of HPAECs and HUVECs

All ECs started from small clumps of cells and spreaded into monolayers within 2 weeks. HPAECs and HUVECs grew slowly at first and the cells divided only at the edge of each clump. After 7 to 10 days in culture, however, cell division was more rapid and became confluent within a matter of days instead of weeks. Unstimulated HPAECs and HUVECs were stained by May-Grünwald Giemsa methods. HPAECs (Fig. 1A) showed some heterogeneity in shape in that at least 10% of the cells were more elongated than HUVECs (Fig. 1B), while both ECs appeared to be of the typical cobblestone morphology in culture.

Factor VIII expression on HPAECs and HUVECs

Both ECs were trypsinized and fluorescently labeled with the antibody to Factor VIII. Figure 2 shows that Factor VIII was expressed on unstimulated HPAECs as well as unstimulated HUVECs at positive cells, 43.1% and 28.9%, respectively.

Expression of CD36 on HPAECs and HUVECs

mAb-recognizing CD36 failed to identify the expression of CD36 on HPAECs and HUVECs by flow cytometric analysis (Fig. 3). Furthermore, the stimulation on HPAECs and HUVECs by TNF-α (10 ng/mL, 6 h), IL-1β (1 ng/mL, 6 h) or LPS (1 μg/mL, 6 h) did not result in the induction of cell surface CD36.

Cellular ELISA analysis of VCAM-1 expression on HPAECs and HUVECs stimulated by TNF-α

VCAM-1 was not expressed on unstimulated HPAECs nor unstimulated HUVECs, but it was induced in a time- and dose-dependent manner on HPAECs and HUVECs stimulated by TNF-α (Figs. 4A and B). VCAM-1 expression was

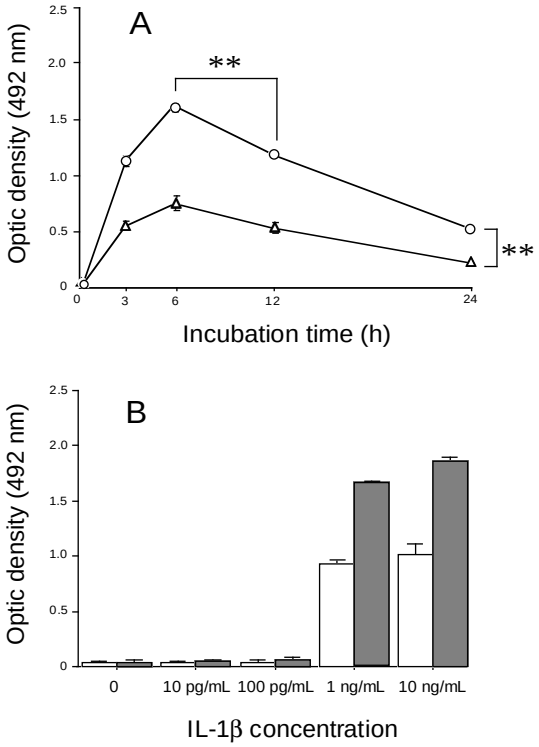

Fig. 5. VCAM-1 expression on HPAECs and HUVECs measured by cellular ELISA after IL-1β stimulation. HPAECs (o) and HUVECs (Δ) in the time-dependent manner were stimulated by IL-1β (1 ng/mL) (**A**). HPAECs (hatched columns) and HUVECs (open columns) in the dose-dependent manner were stimulated by IL-1β for 6 h (**B**). Values are mean ± SD; $n = 5$. Significantly different (**$P < 0.01$) by analysis with two-way ANOVA and the nonparametric Mann-Whitney *U* test between HPAECs and HUVECs in the time-dependent manner.

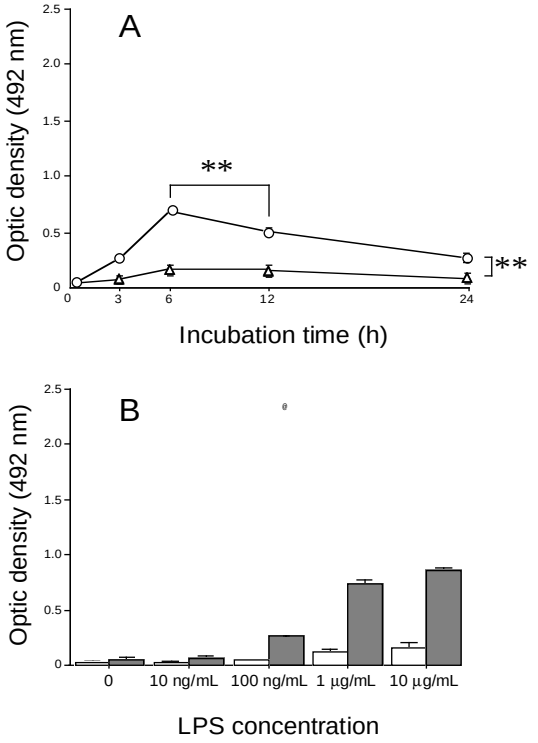

Fig. 6. VCAM-1 expression on HPAECs and HUVECs measured by cellular ELISA after LPS stimulation. HPAECs (o) and HUVECs (Δ) in the time-dependent manner were stimulated by LPS (1 μg/mL) (**A**). HPAECs (hatched columns) and HUVECs (open columns) in the dose-dependent manner were stimulated by LPS for 6 h (**B**). Values are mean ± SD; $n = 5$. Significantly different (**$P < 0.01$) by analysis with two-way ANOVA and the nonparametric Mann-Whitney *U* test between HPAECs and HUVECs in the time-dependent manner.

greater on HPAECs than on HUVECs in a time-dependent manner ($P = 0.009$; two-way ANOVA) (Fig. 4A). After stimulation by TNF-α with 10 ng/mL, VCAM-1 expression on both ECs reached a plateau at 6 h. VCAM-1 expression on HPAECs and HUVECs significantly diminished from the peak level after 24 h ($P = 0.009$; Mann-Whitney *U* test) of TNF-α stimulation, but not after 12 h ($P = 0.251$; Mann-Whitney *U* test). Maximal expression of VCAM-1 was also greater on HPAECs stimulated by TNF-α than on HUVECs ($P = 0.020$; Mann-Whitney *U* test) (Figs. 4B and 7).

Cellular ELISA analysis of VCAM-1 expression on HPAECs and HUVECs stimulated by IL-1β

IL-1β also induced the expression of VCAM-1 on both HPAECs and HUVECs in a time- and dose-dependent manner. There was a significant difference in VCAM-1 expression between both ECs in a time-dependent manner ($P = 0.001$; two-way ANOVA) (Figs. 5A and B). After stimulation by IL-1β with 1 ng/mL, VCAM-1 expression on both ECs reached maximal value at 6 h. VCAM-1 expression on HPAECs and

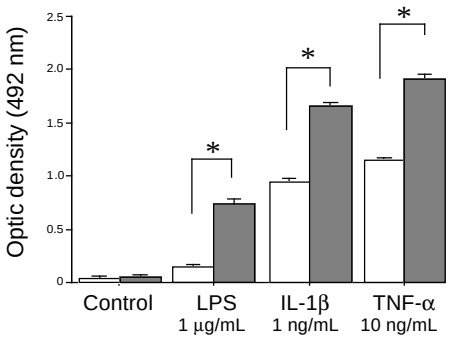

Fig. 7. Comparative effects of VCAM-1 expression on HPAECs (hatched columns) and HUVECs (open columns) after 10 ng/mL TNF-α, or 1 ng/mL IL-1β or 1 μg/mL LPS stimulation for 6 h. Data are expressed as mean ± SD; $n = 5$. Significantly different ($*P < 0.05$) by analysis with the Mann-Whitney unpaired *U* test between HPAECs and HUVECs.

HUVECs after 12 h of IL-1β stimulation significantly diminished ($P = 0.009$; Mann-Whitney *U* test) from the peak level, and thus IL-1β-induced expression of VCAM-1 expression on HPAECs and HUVECs was more transient than TNF-α-induced expression. We also observed that IL-1β was not as potent an inducer of VCAM-1 expression on HPAECs and HUVECs such as TNF-α (Fig. 7). Maximal expression of VCAM-1 stimulation was also greater on HPAECs stimulated by IL-1β than on HUVECs ($P = 0.020$; Mann-Whitney *U* test) (Figs. 5B and 7).

Cellular ELISA analysis of VCAM-1 expression on HPAECs and HUVECs stimulated by LPS

LPS also enhanced VCAM-1 expression on both HPAECs and HUVECs in a time- and dose-dependent manner (Fig. 6A and B). There was also a significant difference in VCAM-1 expression between both the ECs in a time-dependent manner ($P < 0.001$; two-way ANOVA) (Fig. 6A). After stimulation by LPS with 1 μg/mL, VCAM-1 expression on both ECs reached maximal value at 6 h. Although VCAM-1 expression on HPAECs significantly diminished from the peak level after 12 h ($P = 0.009$; Mann-Whitney *U* test) of LPS stimulation, down regulation of VCAM-1 expression on HUVECs was not seen until after 24 h. The stimulation by LPS on HPAECs and HUVECs resulted in low levels of VCAM-1 induction when examined to compare LPS stimulation with IL-1β and TNF-α stimulation (Fig. 7). Maximal expression of VCAM-1 was obviously greater on HPAECs stimulated

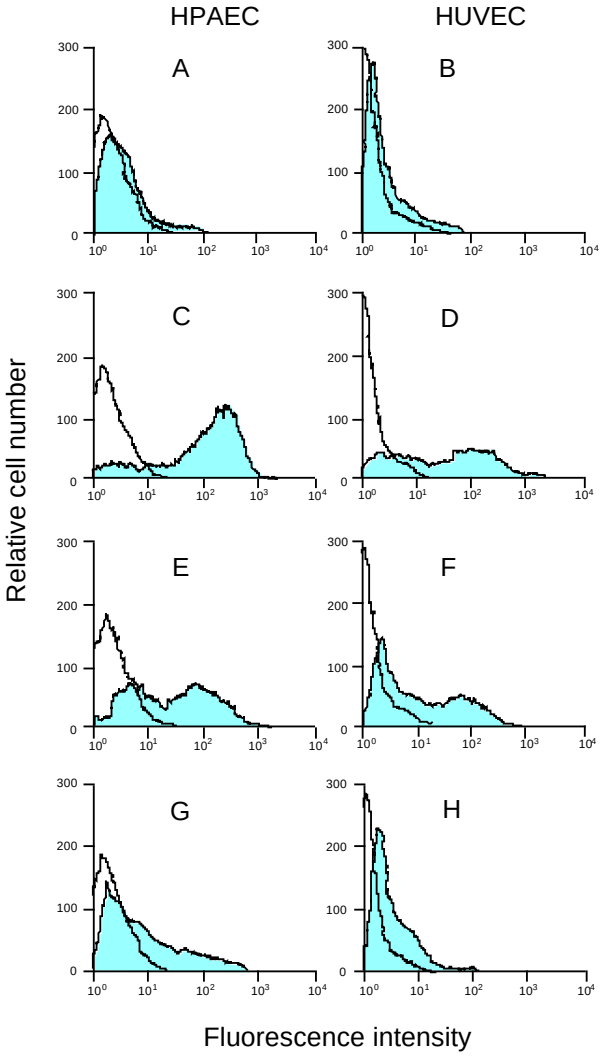

Fig. 8. Flow cytometric analysis of VCAM-1 expression. Unstimulated HPAECs (**A**) and HUVECs (**B**) did not express VCAM-1, but HPAECs (**C**, **E**, **G**) and HUVECs (**D**, **F**, **H**) stimulated with TNF-α (**C**, **D**), IL-1β (**E**, **F**) and LPS (**G**, **H**) induced to express VCAM-1.

by LPS than on HUVECs (P = 0.020; Mann-Whitney U test) (Figs. 6B and 7).

Flow cytometry analysis of VCAM-1 expression on HPAECs and HUVECs

Flow cytometry was performed with 10,000 cells per sample on a FACS analyzer. Fifth passage cells of HPAECs and HUVECs were stained with mAb-recognizing VCAM-1 after trypsinization. HPAECs and HUVECs did not express VCAM-1 at mean fluorescence intensity (MFI; 24.2 and 16.7, respectively), when unstimulated. VCAM-1 expression was significantly greater on HPAECs than on HUVECs when stimulated by 10 ng/mL TNF-α (MFI; 193.0 and 113.8, respectively), 1 ng/mL IL-1β (MFI; 82.4 and 49.1, respectively) or 1 μg/mL LPS (MFI; 28.2 and 18.7, respectively) for 6 h (Fig. 8).

Discussion

We compared HPAECs with HUVECs with regard to VCAM-1 expression induced by TNF-α, IL-1β and LPS. VCAM-1 was expressed greater on HPAECs than on HUVECs stimulated by these cytokines and LPS. Moreover, TNF-α was the most potent signal for VCAM-1 expression on both cell lines.

Both cultured cell lines used in this study were identified as ECs by the expression of Factor VIII on surfaces and by the typical cobblestone morphology in confluent culture, but some HPAECs were more elongated than HUVECs in culture. Both of our cell lines of the 5th passage expressed Factor VIII in 30 to 40% of the positive cells, because the ECs in the growth phase hardly express Factor VIII (Jaffe et al., 1974; Johnson et al., 1980). Moreover, we have demonstrated that CD36, which was reported to be expressed on a majority of microvessels (Tandon et al., 1989; Swerlick et al., 1992b) but not detected on macrovessels (Knowles et al., 1984; Buckley et al., 1985), was not expressed on HPAECs and HUVECs by flow cytometric analysis. So, we examined the differential expression of VCAM-1 between HPAECs and HUVECs as macrovessel cell lines with TNF-α, IL-1β and LPS stimulation.

Our results in a time- and dose-dependent response of VCAM-1 expression on HUVECs stimulated by cytokines and LPS are shown to be consistent with the observation of Carlos and colleagues (1990). We found that the degree of VCAM-1 expression was significantly different at high doses of these stimulators when compared to the degree of expression found between HPAECs and HUVECs by cellular ELISA and flow cytometry. The reason for the difference of the expression on these ECs is not clear. However, these phenomena may be explained at least by the different numbers of surface receptors to cytokines and LPS (Akeson et al., 1992; Lou and Grau, 1993), and the differing regulation of the VCAM-1 gene transcription level. Gille and colleagues (1996) reported that VCAM-1 expression by IL-1 stimulation was lower on human dermal microvascular ECs than on HUVECs, because human dermal microvascular ECs have specific reductions of VCAM-1 gene transcription in response to IL-1. We considered that the ECs from different organs might be diverse in their responses to cytokines and LPS. Similar results to ours have been reported previously. For instance, Hauser and colleagues (1993) also found different levels of VCAM-1 expression on iliac arterial and venous cultured ECs. In binding assays, cells cultured from the bovine pulmonary artery are quite susceptible to the endotoxin effect as polymorphonuclear neutrophil-EC detachments, whereas HUVECs are particularly resistant to binding assays (Staub et al., 1982; Harlan et al., 1983a, 1983b; Meyrick et al., 1989, 1995).

TNF-α was the most potent signal at the peak level and the persistent expression of VCAM-1 on both cell lines. This result was consistent with Swerlick and colleagues (1992a) showing that TNF-α induced more VCAM-1 on HUVECs and human dermal ECs than any cytokines. Although the mechanism of this cytokine to induce VCAM-1 was not known in details, Libby and colleagues (1995) demonstrated that TNF-α could induce IL-1 mRNA in human ECs. We considered that this effect of

autocrine might be attributed to the persistent expression of VCAM-1 by TNF-α.

VCAM-1 is one of the many inducible adhesion molecules that play an important role in the binding of lymphocytes, monocytes and eosinophils to ECs (Weller et al., 1991; Vonderheide and Springer, 1992; Meerschaert and Furie, 1995). Upregulation of the expression of VCAM-1 on ECs is an essential mechanism for the selective accumulation of these inflammatory cells in the lung tissue of patients with asthma (Ohkawa and Yamauchi, 1993; Håkansson et al., 1995). Its regulation has been examined in detail in HUVECs, but it has not been completely investigated in HPAECs. As we demonstrated that VCAM-1 expression was greater in HPAECs than in HUVECs, our results may imply that inflammatory cells more easily migrate into lung tissue. However, in airway inflammation, inflammatory cells themselves take an important part in migrating into lung tissue as well as ECs, and are upregulated to express the ligands to VCAM-1, i.e., CD11b/CD18 and very late antigen-4 by stimulators (Elices et al.,1990; Schwartz et al., 1990; Håkansson et al., 1995). Therefore, when inflammatory models are studied, it is necessary to perform not only quantity assay by cellular ELISA but also binding assay.

In conclusion, we clearly demonstrated that VCAM-1 expression was induced greater on HPAECs than on HUVECs. So, we think that there may exist heterogeneity of ECs obtained from various organs in response to cytokines and LPS. We suggest that HPAECs rather than HUVECs should be used for these experimentations in lung inflammatory models.

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