Interferon-γ and interleukin-4 differentially regulate ICAM-1 and VCAM-1 expression on human lung fibroblasts


ABSTRACT: The expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and more specifically vascular adhesion molecule-1 (VCAM-1) on lung fibroblasts may be important for migration of inflammatory cells through the submucosa to the airway lumen in the asthmatic inflammatory response. This study aimed to assess which cytokines are regulating ICAM-1 and VCAM-1 expression on human lung fibroblasts. For this purpose, confluent fibroblast cultures (derived from lung tissue from a nonasthmatic donor) were stimulated for 4 h with interleukin(IL)-1β, tumour necrosis factor (TNF)α, interferon (IFN)γ, IL-4, IL-5 or transforming growth factor (TGF)β.

IL-1β (optimal concentration (OC) 1 U·mL⁻¹) and TNFα (OC 100 U·mL⁻¹) both increased ICAM-1 and VCAM-1 expression. IFNγ (OC 2 U·mL⁻¹) increased only ICAM-1 expression and IL-4 (OC 5 ng·mL⁻¹) increased only VCAM-1 expression, whereas IL-5 (20 ng·mL⁻¹) and TGFβ (10 ng·mL⁻¹) did not influence ICAM-1 or VCAM-1 expression. ICAM-1 expression reached a plateau at 8–12 h after cytokine stimulation and remained constant for at least 24 h. VCAM-1 showed a transient increased expression within 24 h after IL-1β and TNFα stimulation. In contrast, VCAM-1 expression did not decrease after maximal expression at 4 h upon IL-4 stimulation.

It is concluded that the Helper-1 T-cell type cytokine interferon γ and the Helper-2 T-cell type cytokine interleukin-4 differentially regulate intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on human lung fibroblasts. The proinflammatory cytokines interleukin-1β and tumour necrosis factor α increase both intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression, without differential regulation of the expression of these adhesion molecules.


Keywords: human lung fibroblasts ICAM-1 VCAM-1 cytokines

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IL-1 and TNFα upregulate both ICAM-1 and VCAM-1 expression on endothelial cells [14, 15]. The Th1 type cytokine IFNγ selectively increases ICAM-1 expression [14], whereas the Th2 type cytokine IL-4 selectively increases VCAM-1 expression on endothelial cells [16]. The regulation of ICAM-1 and VCAM-1 expression by cytokines has not yet been fully characterized on human lung fibroblasts.

Therefore, the effects of IL-1β, TNFα, IFNγ, IL-4, IL-5 and TGFβ on the expression of the adhesion molecules ICAM-1 and VCAM-1 on normal human lung fibroblasts were investigated in vitro.

Materials and methods

Study design

To determine the effect of the cytokines IL-1β, TNFα, IFNγ, IL-4, IL-5 and TGFβ on ICAM-1 and VCAM-1 expression of human lung fibroblasts, confluent cultures of these cells were incubated with different concentrations of the aforementioned cytokines and for different periods (ranging 4–24 h). ICAM-1 and VCAM-1 expression were measured using a Cell Surface enzyme linked immunosorbent assay (ELISA) (CSE) technique.

Reagents

Fibroblasts were cultured in Hamˈs F12 medium (Bio-whittaker, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS) (Bodinko BV, Alkmaar, the Netherlands), 125 U·mL⁻¹ Na-penicillin G (Yamanouchi Pharma, Leiderdorp, the Netherlands) and 125 μg·mL⁻¹ streptomycin sulphate (Radiumfarma-Fisiopharma, Milan, Italy). Fibroblasts were passaged by trypsinization with trypsin-cin sulphate (Radiumfarma-Fisiopharma, Milan, Italy) and 125 U·mL⁻¹ o-phenylene diamine dihydrochloride (OPD) was obtained from DAKO A/S (Glostrup, Denmark) and peroxidase (HRP)-conjugated rabbit anti-mouse antibody from CLB (Amsterdam, the Netherlands). Horseradish and mouse IgG1 isotype control antibody was purchased from R&D Systems (Minneapolis, MN, USA), IL-5 (R&D Systems, Abingdon, UK) and IL-4, IL-5 and TGFβ (ranging 4±24 h). ICAM-1 and VCAM-1 expression were determined in a different 96-well plate. For the kinetic experiments, every time point was determined in a different 96-well plate.

Co-stimulation of T lymphocytes for ICAM-1 and VCAM-1 expression on unstimulated fibroblasts.

Fibroblasts were passaged in a ratio of 1:4, grown to confluence (5–8 days, based on microscopical examination) in 96-well culture plates (Costar Europe Ltd., Badhoevedorp, the Netherlands) and used for experiments at passage 5. Fibroblast characterization was performed with antibodies against vimentin, cytokeratin, desmin, smooth muscle actin and fibronectin using fluorescence microscopy. In the used cultures, contamination of fibroblasts with smooth muscle cells was <2%.

Viability of confluent fibroblasts after incubation with different cytokines was assessed using trypan blue exclusion (n=2). In addition, to assess possible heterogeneity between different fibroblast cultures, the experiments were repeated for lung fibroblasts derived from tissue from two other nonasthmatic individuals. Here comparable results concerning ICAM-1 and VCAM-1 upregulation were found.

Cell surface enzyme linked immnosorbent assay

After stimulation with cytokines, fibroblasts in 96-well plates were washed twice with 200 μL cold phosphate buffered saline (PBS) supplemented with 0.01% CaCl₂ and 1% fetal calf serum (FCS) (Bodinko BV, Alkmaar, the Netherlands), and fixed for 10 min in 96% ethanol at 4°C. Subsequently, fibroblasts were air dried for 30 min and stored at 4°C for ≤14 days. CSE was performed using a modified CSE assay according to PIELA and KORN [17]. Briefly, fibroblast layers were incubated with 1% bovine serum albumin (BSA) in PBS for 1 h to block nonspecific binding sites. Then anti-ICAM-1 (2 μg·mL⁻¹), anti-VCAM-1 (10 μg·mL⁻¹) or IgG1 isotype control (4 μg·mL⁻¹) were incubated for 120 min at room temperature, washed according to a 5-step washing procedure performed by a Microplate Strip Washer (Bio-Tek Instruments, Burlington, NJ, USA) and incubated with HRP-conjugated rabbit anti-mouse antibody for 30 min. After the second washing procedure, substrate solution containing OPD was added and colour development was stopped after 30 min by 3 M H₂SO₄. Absorbance at 490 nm was measured using a microplate reader supported by SoftmaxPro software (Molecular Devices, Sunnyvale, CA, USA). A control experiment using 20% human blood group AB serum as blocking agent did not specifically decrease the high VCAM-1 expression on unstimulated fibroblasts.

Analysis of data

ICAM-1 and VCAM-1 expressions are presented as mean optical density (OD) (490 nm) values of quadruplicate determinations within one experiment after subtraction of IgG1 OD values. Outlying OD values within quadruplicates were only left out when they exceeded 2×SD values of the mean; coefficients of variation did not exceed 10%. At least five separate experiments for every cytokine stimulation were performed. To evaluate statistical differences in the concentration experiments, mean OD values representing ICAM-1 and VCAM-1 expressions of separate experiments were tested using the nonparametric Wilcoxon signed rank test for related samples. For the kinetic experiments, every time point was determined in a different 96-well plate. Therefore, an unstimulated control (0 h) in every plate was
Fig. 1 – Effect of different concentrations of cytokines on intercellular adhesion molecule (ICAM)-1 (a, c, e and g) and vascular cell adhesion molecule (VCAM)-1 (b, d, f and h) expression of human lung fibroblasts after 4 h stimulation. Results are presented as mean optical density (OD) 490 nm values after subtraction of isotype control. ■: two separate experiments; ●: five separate experiments. *: p<0.05 compared to baseline; #: p<0.05 compared to lower adjacent concentration (Wilcoxon signed rank). IL: interleukin; TNFα: tumour necrosis factor-α; IFNγ: interferon-γ.
assessed. For evaluation of differences in ICAM-1 and VCAM-1 between different time points, mean OD values of stimulated wells subtracted with OD values of the unstimulated control within the same plate were used. Differences were considered significant at p<0.05.

**Results**

**Concentration-dependent increase of intercellular adhesion molecule-1 and/or vascular adhesion molecule-1 expression after cytokine stimulation**

Expression of ICAM-1 and VCAM-1 on human lung fibroblasts was differentially regulated after stimulation with various cytokines. IL-1β and TNFα each significantly increased both ICAM-1 and VCAM-1 expression on human lung fibroblasts after 4 h in a concentration-dependent way. Maximal expression of ICAM-1 and VCAM-1 was achieved with 1 U·mL⁻¹ IL-1β (fig. 1a and b; increase 6- and 1.5-fold compared to baseline, respectively). Maximal ICAM-1 expression was reached with 100 U·mL⁻¹ TNFα and maximal VCAM-1 expression with 10 U·mL⁻¹ (fig. 1c and d; increase 3- and 1.5-fold compared to baseline, respectively). IFNγ strongly and selectively increased ICAM-1 expression dose-dependently. Maximal ICAM-1 expression was reached with 2 U·mL⁻¹ IFNγ (fig. 1e and f; increase 6-fold compared to baseline). In contrast to IFNγ, IL-4 selectively increased VCAM-1 expression in a concentration-dependent manner, 0.5 ng·mL⁻¹ being significantly less potent (p<0.05) than 5 ng·mL⁻¹ (fig. 1g and h; increase 1.5-fold compared to baseline). Finally, IL-5 (0.2, 2, 20 ng·mL⁻¹) and TGFβ (1, 100 pg·mL⁻¹, 10 ng·mL⁻¹) did not influence ICAM-1 or VCAM-1 expression after 4 h (data not shown). Comparable results were found 24 h after stimulation with the above mentioned cytokines, apart from the fact that VCAM-1 expression did not change compared to baseline after IL-1β stimulation, and a significantly reduced VCAM-1 expression occurred after 100 pg·mL⁻¹ and 10 ng·mL⁻¹ TGFβ stimulation (fig. 2; decrease by a factor 2 compared to baseline). None of the cytokines reduced fibroblast viability below 95% after 4 or 24 h (not shown).

**Time-dependent increase of intercellular adhesion molecule-1 and/or vascular cell adhesion molecule-1 expression after cytokine stimulation**

The kinetics of ICAM-1 and VCAM-1 expression after activation of human lung fibroblasts with different cytokines was investigated, using one representative cytokine concentration and was monitored over a time period of 24 h with 4 h intervals. It was found that 1 U·mL⁻¹ IL-1β stimulation at all time points, significantly increased ICAM-1 expression (fig. 3a) and VCAM-1 expression (fig. 3b) (p<0.05) compared to baseline. Significant differences between ICAM-1 expressions after 4 and 8 h, as well as 8 and 12 h (p<0.05) indicated a plateau after 12 h of stimulation. VCAM-1 expression was transiently increased reaching a maximum at 8 h, followed by a significant decrease after 20 and 24 h of stimulation (p<0.05).

TNFα (1 U·mL⁻¹) significantly increased ICAM-1 (fig. 3c) and VCAM-1 expression (fig. 3d) at all time points (p<0.05), except for ICAM-1 at 12 h, where there was a trend to increased expression (p=0.08). ICAM-1 expression reached a plateau at 8 h and VCAM-1 expression was maximal at 12 h and was significantly decreased after 20 h of stimulation.

IFNγ (2 U·mL⁻¹) significantly increased ICAM-1 expression at all time points (fig. 3e) and a plateau level was reached after 12 h of stimulation. In general, VCAM-1 expression was not affected by IFNγ stimulation (fig. 3f). A small significant increase in VCAM-1 expression was found after 20 h of IFNγ stimulation.

IL-4 stimulation did not influence ICAM-1 expression within 24 h (fig. 3g), but IL-4 (0.5 ng·mL⁻¹) significantly increased VCAM-1 expression on fibroblasts at all time points (p<0.05), except at 16 h (fig. 3h). No significant differences were found between VCAM-1 expressions at different time points, indicating a maximum expression already at 4 h and no decrease over time. It is important to note that similar results were obtained using fibroblast cultures from two other nonasthmatic subjects (not shown).

**Discussion**

This study demonstrates that proinflammatory IL-1β and TNFα increase ICAM-1 and VCAM-1 expression on human lung fibroblasts isolated from a nonasthmatic individual. IFNγ primarily increases ICAM-1 expression, whereas IL-4 only increases VCAM-1 expression. IL-5 does not affect ICAM-1 or VCAM-1 expression on human lung fibroblasts, whereas TGFβ decreases VCAM-1 expression after 24 h. The differential upregulation of these adhesion molecules by Th1 and Th2 cytokines may add to increased infiltration of inflammatory cells in the pulmonary tissue as seen in asthma by binding to the corresponding adhesion molecules expressed on, e.g., eosinophils.
Fig. 3 – Time-dependent effect of different cytokines on intercellular adhesion molecule (ICAM)-1 (a, c, e and g) and vascular cell adhesion molecule (VCAM)-1 (b, d, f and h) expression of human lung fibroblasts. Results are presented as mean optical density (OD) 490 nm values after subtraction of isotype control. a and b) 1 U·mL⁻¹ interleukin (IL)-1β (six separate experiments); c and d) 1 U·mL⁻¹ transforming growth factor (TNF)α (five separate experiments); e and f) 2 U·mL⁻¹ interferon (IFN)γ (seven separate experiments); g and h) 0.5 ng·mL⁻¹ IL-4 (seven separate experiments). *: p<0.05 compared to baseline; #: p<0.05 compared to maximal expression (Wilcoxon signed rank).
Complement receptor 3 (CR3) (ligand to ICAM-1) is expressed by both eosinophils and neutrophils at a high level. In contrast, VLA-4 (ligand to VCAM-1) is highly expressed on eosinophils, whereas neutrophils express VLA-4 at very low levels. Therefore, VCAM-1 expression may specifically cause eosinophil infiltration when binding to VLA-4 occurs [18, 7].

The increase in OD490 nm of ICAM-1 expression on fibroblasts was generally higher than of the corresponding VCAM-1 expression at 4 h, both with IL-1β (1 U·mL⁻¹) and TNFα (100 U·mL⁻¹) stimulation (fig. 1a–d). This does not necessarily imply that ICAM-1 is more strongly upregulated than VCAM-1, because the authors were not able to use a standard curve for the amount of cellular adhesion molecules in the CSE. The lower VCAM-1 upregulation can also be due to the relatively high baseline VCAM-1 expression found with all fibroblast cultures. This high baseline expression for VCAM-1 could probably not entirely be ascribed to nonspecific binding, because high concentrations of human blood group AB serum were also unable to specifically block anti-VCAM-1 binding. The variability of OD values between the separate experiments can be ascribed to unknown differences in cell culture conditions. It was found that VCAM-1 expression was most susceptible to variations between separate experiments. Additional fluorescence-activated cell sorter (FACS) experiments revealed similar patterns of upregulation of ICAM-1 and VCAM-1 expression by IL-1β, TNFα, IFNγ and IL-4 at 4 and 24 h as with CSE (not shown), with a similar selectivity for the IFNγ-induced ICAM-1 and IL-4-induced VCAM-1 expressions.

Pro-inflammatory cytokines increase both ICAM-1 and VCAM-1 expression. IL-1β maximally increases ICAM-1 and VCAM-1 expression at a concentration of 1 U·mL⁻¹. TNFα is less potent than IL-1β and maximally increases ICAM-1 expression after stimulation with 100 U·mL⁻¹ and VCAM-1 expression with 10 U·mL⁻¹. The current observations after IL-1β and TNFα stimulation are in accordance with studies showing that IL-1β and TNFα are able to increase ICAM-1 and VCAM-1 expression on skin and synovial fibroblasts [19–21]. This study has extended these findings in that the cytokines IFNγ and IL-4 showed a differential pattern of increasing ICAM-1 and VCAM-1 expression on human lung fibroblasts. IFNγ maximally increased ICAM-1 expression at a concentration of 2 U·mL⁻¹ after 8 h and did not decrease within 24 h. Moreover, a significant, but small increased expression of VCAM-1 after 20 h of stimulation occurred, implying that IFNγ may stimulate VCAM-1 expression after an extended incubation time. However, at 24 h of stimulation, there was no significant increase. On endothelium and skin fibroblasts, IFNγ increased ICAM-1 expression [21, 17]. In addition, Gao and Issekutz [20] found increased VCAM-1 expression on skin fibroblasts at 20–24 h after IFNγ stimulation, as well as an increased VCAM-1 messenger ribonucleic acid (mRNA) formation. These results after IFNγ stimulation together suggest an early onset of increased ICAM-1 expression, while an increased VCAM-1 expression may be seen at later stages of IFNγ stimulation.

IL-4 maximally increased VCAM-1 expression at a concentration of 5 ng·mL⁻¹ after 4 h of stimulation. No decline in VCAM-1 expression within 24 h was observed. These findings are consistent with findings on endothelium [16], lung fibroblasts [21] and skin fibroblasts [22] on which IL-4 selectively upregulated VCAM-1 expression. However, on skin fibroblasts, Piela-Smith et al. [23] found an increase in ICAM-1 expression after 4 h of IL-4 stimulation (100 ng·mL⁻¹), suggesting that skin fibroblasts may react differently from lung fibroblasts upon IL-4 stimulation. IL-4 was not able to increase ICAM-1 expression on human lung fibroblasts in the current study.

The results show that increased VCAM-1 expression is transient within 24 h after IL-1β and TNFα stimulation, in contrast to the kinetics of increased ICAM-1 expression. The dichotomy in the kinetics of VCAM-1 expression on lung fibroblasts after IL-1β and TNFα stimulation on the one hand (transient within 24 h) and IL-4 stimulation on the other (constant within 24 h) is conspicuous and cannot easily be explained. It is known that IL-1β and TNFα use different signal transduction pathways than IL-4. For VCAM-1 expression in endothelial cells, IL-4 acts via the Signal transducer and activator of transcription-6 (STAT6) protein [24]. TNFα and probably IL-1β use transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1 [25, 26]. The underlying mechanism of the increase followed by a decrease in VCAM-1 expression within 24 h of IL-1β and TNFα stimulation is not completely understood. It has been described before on vascular smooth muscle cells that the stability of VCAM-1 expression after TNFα stimulation is much lower than the stability of ICAM-1 expression [27]. The removal of a membrane molecule can be explained either by internalization or by shedding. Shedding seems to be the most likely mechanism, because soluble VCAM-1 (sVCAM-1) has been detected after stimulation of endothelium, and there are indications for proteolytic cleavage of a truncated VCAM-1 isoform in mouse endothelial cells [28]. The persistent VCAM-1 level after IL-4 stimulation suggests that such shedding is not activated by IL-4 within 24 h. The expression of VCAM-1 by the cytokines IL-1β, TNFα and IL-4 may affect the kinetics of eosinophil migration into the pulmonary tissue in allergic inflammation and adds the fibroblast as a regulating intermediate cell to the endothelial and epithelial cells.

Two other cytokines known to play an important role in the process of asthma were assessed for their influence on ICAM-1 and VCAM-1 expression on human lung fibroblasts. IL-5 did not influence ICAM-1 or VCAM-1 expression on lung fibroblasts, in contrast to its fellow Th2 cytokine IL-4. A possible explanation is that fibroblasts do not express IL-5 receptors and therefore no signal transduction route is activated. Generally the IL-5 receptor is expressed on leukocytes and possibly not on the constituent cells of the lung tissue. Comparably, stimulation of the human bronchial epithelial cell line BEAS-2B with IL-5 also failed to influence ICAM-1 and VCAM-1 expression [29].

TGFβ did not influence ICAM-1 expression, but downregulated VCAM-1 expression on lung fibroblasts after 24 h. This phenomenon has been shown before on bone marrow fibroblasts [30], but not on microvascular endothelium [31]. TGFβ is known to act primarily on fibroblasts. The downregulation of VCAM-1 at 24 h in our study might be an effect of a changing phenotype of fibroblasts after TGFβ stimulation: there are indications that TGFβ has the capacity to convert fibroblasts to the alpha smooth
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References

15. Carlos TM, Schwartz BR, Kovach NL, et al. Vascular cell adhesion molecule-1 and vascular cell adhesion molecule-1 expression, respectively. interleukin-5 and interleukin-4 differentially and specifically influence intercellular adhesion molecule-1 nor vascular cell adhesion molecule-1 expression. The selective increase of vascular cell adhesion molecule-1 on lung fibroblasts by the Th2 type cytokine interleukin-4 may contribute to selective eosinophil transmigration through the pulmonary tissue in a directional migration to the epithelial cells. The finding that fibroblasts possibly act as important intermediate cells in the inflammatory response, places these cells in focus for modulation by therapeutic regimens currently used in asthmatic patients.

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The knowledge about factors increasing intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on human lung fibroblasts is limited. This study shows that interleukin-1β and tumour necrosis factor α are able to increase both intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on normal human lung fibroblasts, while interferon γ and interleukin-4 differentially and specifically influence intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression, respectively. interleukin-5 and transforming growth factor β neither increased intercellular adhesion molecule-1 nor vascular cell adhesion molecule-1 expression. The selective increase of vascular cell adhesion molecule-1 on lung fibroblasts by the Th2 type cytokine interleukin-4 may contribute to selective eosinophil transmigration through the pulmonary tissue in a directional migration to the epithelial cells. The finding that fibroblasts possibly act as important intermediate cells in the inflammatory response, places these cells in focus for modulation by therapeutic regimens currently used in asthmatic patients.


