Original Article

Cytokine Responsiveness in Cultured Human Small Airway Epithelial Cells in Relation to Lung Transplantation

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Background: **We report a new method for collecting and establishing small airway epithelial cells (SAEC). This method enables the evaluation of the cytokine responsiveness of SAEC, which is still unknown. In this study we evaluated intercellular adhesion molecule-1 (ICAM-1) expression on SAEC stimulated with several inflammatory cytokines and compared it with that on large airway epithelial cells (LAEC).**

Materials and Methods: **LAEC and SAEC were treated with IFN-γ, TNF-α, IL1-β, or their combination. ICAM-1 expression under various conditions was quantified by flow cytometry. Furthermore, immunocytochemical staining was performed to determine intranuclear displacement of signal transducer and activator transcription 1 (Stat1) during ICAM-1 expression by various cytokine stimulations.**

Results: **1) ICAM-1 expression on both LAEC and SAEC was significantly increased by IFN-γ stimulation alone and synergistically enhanced by IFN-γ plus TNF-α or IL-1β stimulation, 2) intranuclear displacement of Stat1 in SAEC by the stimulation with IFN-γ plus TNF-α or IL-1β was recognized earlier in comparison with that by IFN-γ stimulation alone.**

Conclusion: **The previously unknown peripheral cytokine responsiveness and its mechanisms of SAEC were revealed by this study, which contributes to the understanding of chronic lung allograft rejection recognized around small airways. (Ann Thorac Cardiovasc Surg 2005; 11: 374–81)**

Key words: small airway epithelial cells, large airway epithelial cells, intercellular adhesion molecule-1, signal transducer and activator transcription 1, bronchiolitis obliterans

Introduction

Initial optimism engendered by early successes in lung transplants has been tempered by the development of histologic bronchiolitis obliterans (BO) or of the progressive airway obstruction called BO syndrome, over the long term.[1)] The final common pathway of all inciting events seems to be an alloimmune injury, with subsequent release of immunologic mediators and production of growth factors, leading to luminal obliteration and fibrous scarring of small airways. Bronchiolitis is a generic term that encompasses a group of diseases with diverse etiologies.[2,3)] In general, it indicates the presence of inflammation in the small airways, which by definition are airways that measure 2 mm or less in diameter. The causes of bronchiolitis can be classified on clinical grounds, by etiology or by histologic appearance. A possible mechanism for the induction of lung allograft rejection is the inflammatory up-regulation of adhesion molecules on the en-

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dothelium of the transplanted organ, followed by T cell activation. The mechanisms of lung allograft rejection have been studied with respect to intercellular adhesion molecule-1 (ICAM-1). ICAM-1 expression has been confirmed on human vascular endothelial cells and on large airway epithelial cells (LAEC). However, primary human small airway epithelial cells (SAEC) must be involved in the mechanisms of lung allograft rejection, because the small airways are the place where leukocytes from the blood stream actually start interacting with bronchial epithelial cells. Therefore, we established a method for collection and culture of SAEC. First of all, the cytokine responsiveness to ICAM-1 expression was compared in LAEC and SAEC. Cytokine responsiveness with respect to immunity, inflammation and lung allograft rejection often depends on the transcription factors termed the signal transducer and activator of transcription 1 (Stat1), so Stat1 signal pathways are candidates for involvement in lung allograft rejection.

Bronchial epithelial cell monolayers were grown to 90% confluence on 24-chamber slides and then were treated with or without IFN-γ or TNF-α, IL-1β or their combinations. ICAM-1 expression on bronchial epithelial cells was analyzed by flow cytometry. In addition, the intranuclear displacement of Stat1 was evaluated using immunofluorescent cytochemistry.

Materials and Methods

Harvesting of airway epithelial cells

Five patients with no evidence of interstitial lung disease were studied (5 men; mean age 62 years; range 44 to 75 years). Four of the patients were smokers. All underwent routine bronchoscopy on the suspicion of bronchial carcinoma. In all of them, before lavage the lung was roentgenographically normal and the brushing cytology showed a normal differential cell count. Fully informed consent for the procedure was obtained from all patients.

The large airways and small airways (1.7 mm or less in diameter) were examined as carefully as possible. Endobronchial brushing was performed at the trachea for LAEC and at a peripheral site of the middle or lower lobe bronchus for SAEC. SAEC was collected by using a ϕ 1.0 mm small diameter brush (BC-14C, Olympus Optical Co., Ltd., Tokyo, Japan) covered with a sheath, which was designed to prevent contamination, and theoretically this type of brush can be inserted to pass through the small airways (Fig. 1).

Epithelial cells were harvested by shaking the brush in culture medium (ice-cold RPMI-1640 supplemented with 10% FCS, amphotericin B and penicillin/streptomycin). The medium was immediately centrifuged for 5 min at 1,000 rpm, then washed with cold phosphate-buffered saline (PBS) and subjected to hypotonic lysis to eliminate red blood cells. Brushing specimens obtained using this method contained 80-90% viable cells by the trypan blue exclusion method. The numbers of cells were counted (total number of cells: 10^4-10^5) and the concentration was adjusted to 1×10^4 cells/ml. Aliquots of the cell suspension were subjected to cytocentrifugation onto glass slides followed by fixation with methanol for 5 min at 20°C, and slides were air dried and stored at 4°C for subsequent immunostaining. Airway epithelial cells from all subjects comprised more than 95% of the brushed cells, based on morphology and immunostaining with anti cytokeratin (Cosmo Bio Co., Ltd., Tokyo, Japan) and anti-vimentin (DakoCytomation A/S, Glostrup, Denmark) mAb (Fig. 2).

Airway epithelial cell culture

Primary cultures of SAEC and LAEC were established from patients who had no clinical history of airway infection. The airway epithelial cells were cultured in small airway growth media (SAGM; Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA), supplemented with bovine pituitary extract (15 mg/ml), human epithelial growth factor (0.25 μg/ml), epinephrine (0.25 μg/ml), hydrocortisone (0.25 mg/ml), transferrin (5 mg/ml), insulin (2.5 mg/ml), retinoic acid (0.05 μg/ml), triiodotyronine (3.25 μg/ml), gentamicin (25 mg/ml), amphotericin-B (25 μg/ml), and BSA-FAF (25 mg/ml). For experimental use, second passage cells were plated on tissue culture-treated collagen coated 24-well plates (Collagen coated type I; Asahi Techno Glass Co., Tokyo, Japan) and cultured until confluence at 37°C.

Cytokine stimulation conditions

Concentration estimates of ICAM-1 expression after IFN-γ (Biosciences Pharmingen, San Diego, CA, USA) stimulation (0.1-1,000 U/ml) were carried out by applying IFN-γ to confluent epithelial monolayers. An IFN-γ concentration that produced physiologically relevant expression levels of ICAM-1 expression (100 U/ml) was chosen. In addition, time-course responses experiments measuring ICAM-1 expression (6, 12, 18, 24 hrs) in response to 100 U/ml of IFN-γ stimulation were performed, and a suitable incubation time (24 hrs) was chosen.

ICAM-1 expression on airway epithelial cells

To evaluate ICAM-1 expression on epithelial cells, mono-

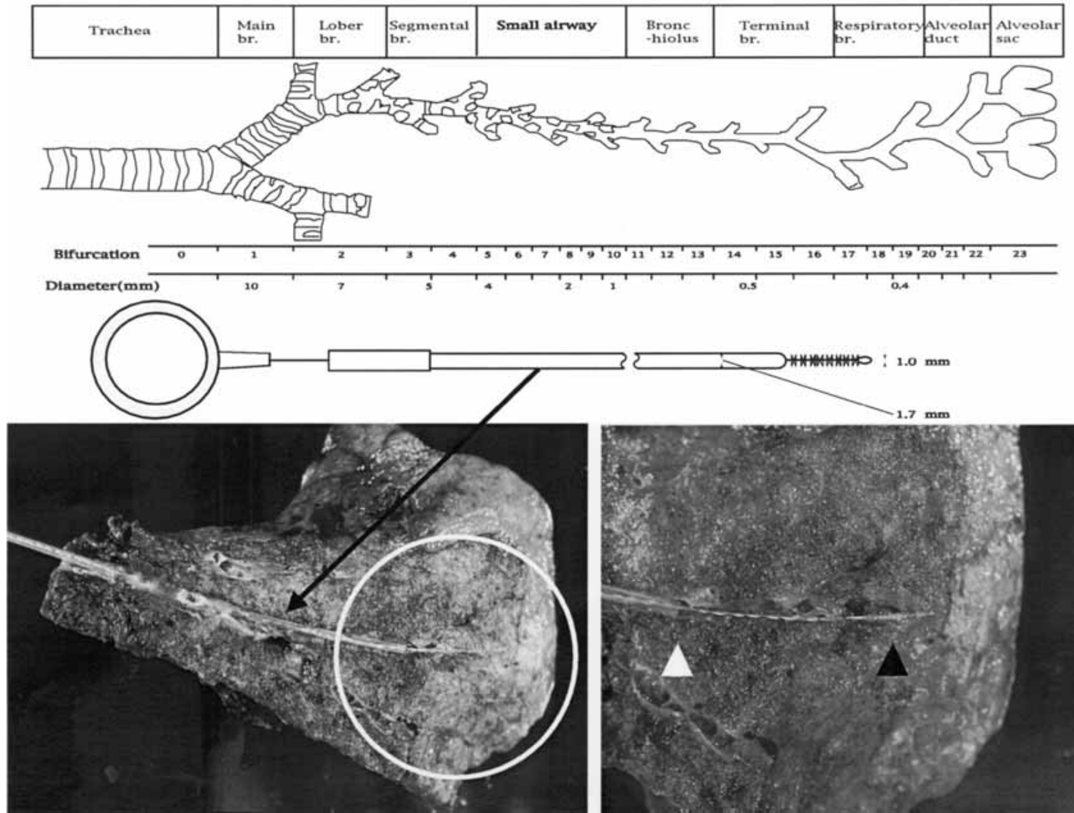

Fig. 1. The tip of BC15-C has an outer diameter of 1.0 mm extended straight from the wedge of the covered sheath (external diameter of 1.7 mm: △). It is fitted with a short firm brush to collect cells from the small airway in the right middle lobe resected from a patient of lung cancer (1.0 mm diameter brush: ▲).

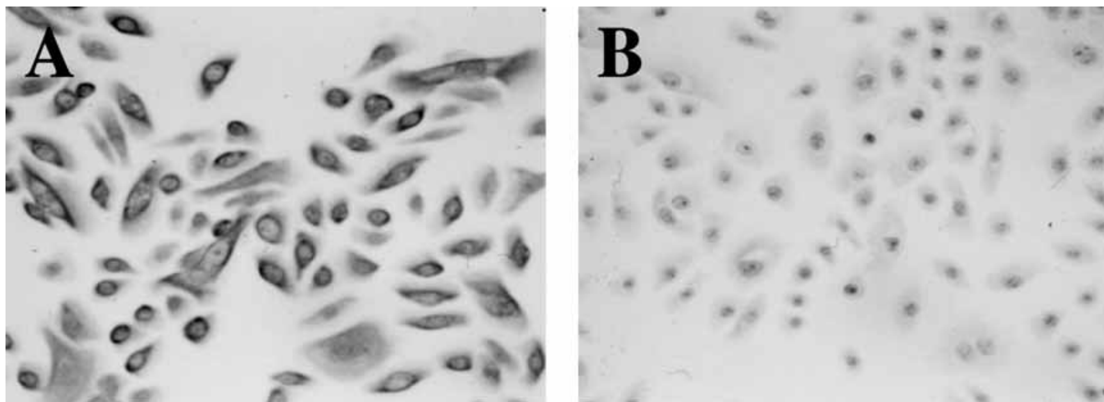

Fig. 2. SAEC monolayers were grown to 50% confluency on 8-Well CultureSlides (Rat Tail Collagen, Type I; BIOCOAT CELLWARE: Beckman Coulter, Inc.). Indirect immunoperoxidase staining with anti cytokeratin (A) and vimentin (B) mAb resulted in a positive staining for cytokeratin and negative staining for vimentin antigens. (Magnification: ×400)

layers were grown to 70% confluence and either untreated or treated with 100 U/ml IFN-γ, TNF-α and IL-1β (Sigma, St. Louis, MO, USA) or a combination of any two of them for 24 hrs. After cytokine stimulation, cells were washed with PBS, harvested by treatment with 0.05% trypsin/EDTA (Invitrogen Corp., Grand Island, NY, USA), and finally resuspended in binding buffer (0.2% BSA in PBS) containing fluorescein isothiocyanate

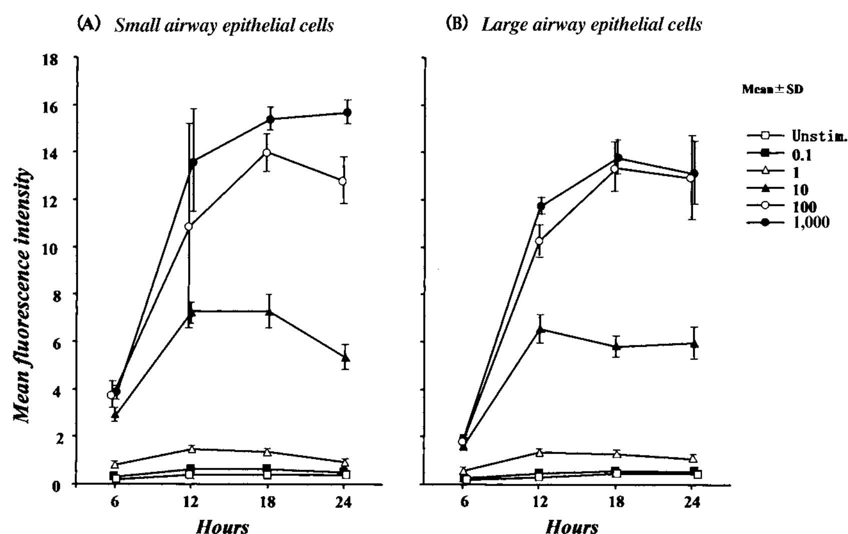

Fig. 3. Concentration responses for stimulation of ICAM-1 expression in SAEC (A) and LAEC (B) cell monolayers. In A and B, monolayer (5×10^5) was studied unstimulated or with IFN-γ, TNF-α, IL-1β, stimulation (0.1-1,000 U/ml for 24 hrs at 37°C), and cellular levels of ICAM-1 were assayed using anti-ICAM-1 Mab LB-2 as primary antibodies and FITC-conjugated goat anti-mouse IgG in a flow cytometry-based immunoassay. Values represent means±SD (n=5) and are representative of 3 experiments.

(FITC)-conjugated mouse anti-ICAM-1 mAb (Beckman Coulter, Inc., Fullerton, CA, USA) for 1 hr at 4°C for flow cytometrical analysis. Fluorescence was measured using a Coulter Epics XL Flow Cytometer (Beckman Coulter, Inc.) and was expressed as a percent age of control mean fluorescence intensity. For each sample at least 10,000 events were collected, and histograms were generated. A combination of negative staining with propidium iodide (2 μl/ml) and scatter characteristics was used to identify a uniform population of viable cells.

Immunostaining for Stat1

Stat1 (R&D Systems, Inc., Minneapolis, MN, USA) is one of the cytokine signal transduction pathways that activates the genetic transcription of the IFN-γ responsive gene (after chronological stimulation at 5, 10, 15, 20 min). Stat1 was evaluated by immunostaining to confirm synergistic enhancement of ICAM-1 by stimulation of the cytokine. SAEC were grown to 80% confluence on 8-well chamber slides (Beckman Coulter, Inc.), and treated with or without 100 U/ml of IFN-γ, TNF-α, IL-1β or a combination of any two of them for 5-20 min.

After cytokine stimulation, the cells were rinsed three times with PBS and fixed with methanol for 5 min, then incubated with PBS containing 2% albumin for 20 min at room temperature to block nonspecific immunoglobulin binding. Goat anti-human Stat1 p91(DAKO A/S; Glostrup, Denmark) and 2% albumin in PBS was added (1/50 final dilution), and the slides were incubated for 1 hr at room temperature. Next, the cells were washed with PBS and treated with FITC-conjugated rabbit anti-goat IgG (1/100 final dilution in PBS with 2% albumin) for 40 min at room temperature. Slides were mounted and viewed using an epifluorescence photomicrography system set for a 15-second exposure (magnification: ×200) and Kodak T-Max 100 film.

Statistical analysis

Student's *t* test was used for comparison between the groups, and a p value of less than 0.05 was considered to indicate a statistically significant difference.

Results

ICAM-1 expression on airway epithelial cells

ICAM-1 expression was significantly enhanced with 1 ng/ml of IFN-γ (ICAM-1 expression without stimulus, $p<0.05$). TNF-γ and IL-1β had no effect on ICAM-1 expression.

IFN-γ alone induced a small but significant increase in ICAM-1 expression. Data in Fig. 3 show the concentration dependence of ICAM-1 expression by cytokines on SAEC. A significant ($p<0.05$) increase in ICAM-1 expression over baseline levels was observed with concentrations of IFN-γ as low as 1 U/ml. Induction reached a plateau at a dose of 100 U/ml of IFN-γ. With regard to findings, ICAM-1 expression began to increased to a plateau that lasted from 18 to 24 hrs after inoculation (Fig. 3).

Synergistic effect of IFN-γ with TNF-α and IL-1β on airway epithelial cells

IFN-γ (100 U/ml), either alone or in combination with TNF-α (100 U/ml) or IL-1β (100 U/ml), induced ICAM-1 expression, as indicated by SAEC flow cytometry. In-

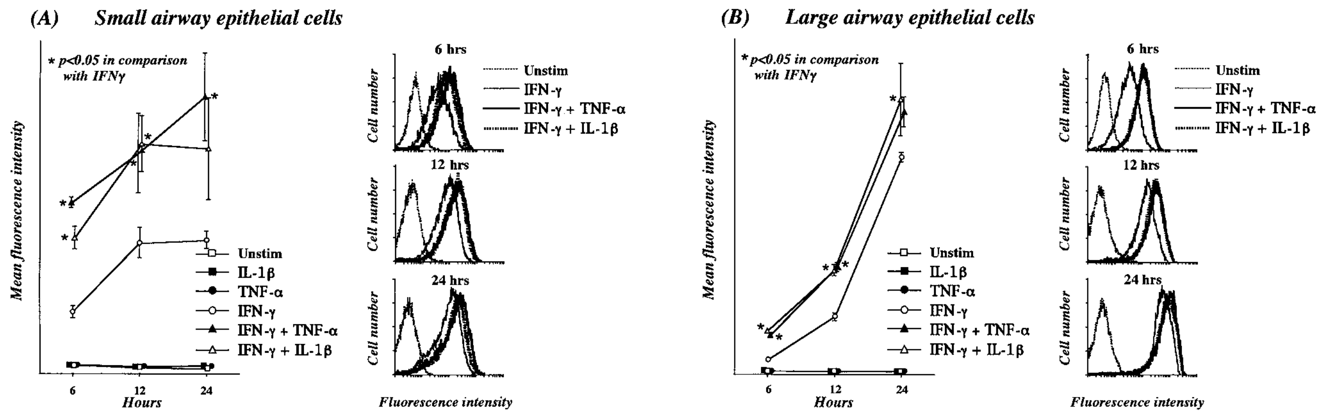

Fig. 4. Effect of activator concentration on ICAM-1 expression on SAEC (A) and LAEC (B) pretreated with IFN-γ. SAEC and LAEC were cultured medium alone, 100 U/ml IFN-γ alone and 100 U/ml TNF-α or 100 U/ml IL-1β with 100 U/ml IFN-γ. The level of ICAM-1 expression was determined by flow cytometory, as described in Materials and Methods.
*Significant increase from 100 U/ml IFN-γ alone level.

cubation with IFN-γ plus TNF-α or IL-1β resulted in a greater enhancement of ICAM-1 expression over the response induced by IFN-γ alone for 6-24 hrs ($p<0.05$). The combination of 100 U/ml of IFN-γ plus either TNF-α or IL-1β increased ICAM-1 expression in LAEC after 24 hrs of incubation (Fig. 4). TNF-α or IL-1β alone did not enhance ICAM-1 expression. A representative fluorescence histogram of ICAM-1 expression induced by IFN-γ is shown in Fig. 3. Staining patterns of ICAM-1 were bimodal with a progressively larger proportion of the cells staining positively as the concentration of IFN-γ increased.

Comparison of Stat1 expression on SAEC after different treatments

Stat1 translocation was assessed by immunofluorescence microscopy of SAEC monolayers that were either untreated or treated with IFN-γ plus TNF-α or IL-1β for 5-20 min. Stat1 was detected using an anti-Stat1 primary Ab and a FITC-conjugated secondary Ab. Monolayers either treated, untreated or with TNF-α (100 U/ml) or IL-1β (100 U/ml) for 20 min plus IFN-γ (100 U/ml) for 5 min did not significantly affect Stat1 phosphorylation, because IFN-γ-induced Stat1 phosphorylation rates remain steady during the early stage stimulation by IFN-γ (i.e., nuclear translocation and DNA binding).

Using immunofluorescence cytochemistry to assess nuclear translocation, we found that Stat1 translocation does not occur during the early stage (at 5 min) of treatment with IFN-γ alone, but it was seen during the late stage (at 20 min) stimulation. However, when IFN-γ was combined with either TNF-α or IL-1β, nuclear translocation was seen even after only 5 min (Fig. 5).

Based on data from isolated SAEC, nuclear localization appears to represent an accurate indicator of Stat1 activation and Stat1-dependent gene transcription. Each of these findings (together with data on airway epithelial cells and Stat1-dependent cell lines showing that Stat1 activation is sufficient for ICAM-1 gene transcription and expression)[4-7] indicates that Stat1 activation is likely linked to Stat1-dependent expression of its target gene (ICAM-1) in SAEC.

IFN-γ is the only cytokine that we have found to activate both Stat1 and ICAM-1 gene expression in SAEC and LAEC. Similarly, IFN-γ treatment of bronchial epithelial cells brushed from airways of normal controls results in increased Stat1 nuclear translocation, and end bronchial biopsies of these subjects exhibited increased Stat1 translocation and ICAM-1 expression.[8]

Discussion

The vascular endothelium controls the recruitment of leukocytes into tissues through the induction and modulation of leukocyte-selective endothelial cell adhesion and migration pathways. Leukocyte recruitment from the blood involves multiple steps: an initial contact or rolling step, mediated by primary adhesion receptors; chemokine or chemoattractant activation of secondary adhesion receptors; firm attachment; and transendothelial migra-

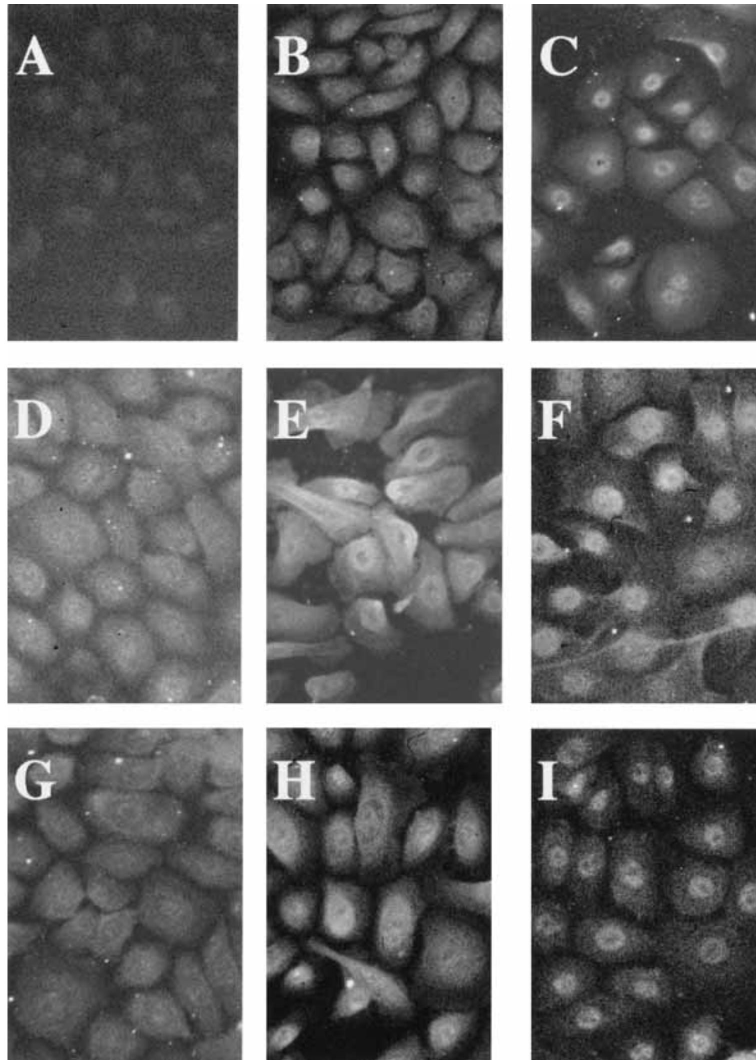

Fig. 5. Fluorescence photomicrographs of unstimulated and cytokine-stimulated SAEC monolayers immunostained for Stat1. Nuclear localization of the Stat1 in SAEC treated with or without IFN-γ (100 U/ml) and a combination of TNF-α (100 U/ml) or IL-1β (100 U/ml). No fluorescence was detected for monolayers stained with nonimmune IgG or with only anti-mouse IgG. Unstimulated (A) and IFN-γ treated cells for 5 (B) and 20 min (C), TNF-a treated cells for 20 min (D), IFN-γ and TNF-α treated cells for 5 (E) and 20 min (F), IL-1β treated cells for 20 min (G), IFN-γ and IL-1β treated cells for 5 (H) and 20 min (I). (Magnification: ×200)

tion.[9,10] Cytokines that are known to be released soon after viral infection,[11-13] such as IFN-γ, increase ICAM-1 expression on LAEC.

Epithelial cell derived factors enhancing ICAM-1-mediated adherence of neutrophils and eosinophils to epithelial cells have been identified as TNF-α and IL-1β.[14] Individually TNF-α and IL-1β did not increase ICAM-1 expression by SAEC, but in combination with IFN-γ both enhanced expression.

Published results of studies of ICAM-1 expression on bronchial epithelial cells induced by cytokines are conflicting. Look and coworkers found that IFN-γ, but not TNF-α or IL-1β, enhanced ICAM-1 expression on human tracheal epithelial cells in primary culture.[14] Bloemen and coworkers also found that IFN-γ, but not TNF-α, enhanced ICAM-1 expression on the NCI-H292 cell line.[15] In contrast, both TNF-α and IL-1β were found by Wegner and coworkers to enhance ICAM-1 expression in a dose-dependent manner on cultured monkey bronchial epithelial cells.[16] It is likely that ICAM-1 expression under baseline conditions or upon stimulation with cytokines plays an important role in the adhesion of leukocytes. Godding and coworkers reported that ICAM-1 did not mediate eosinophil adhesion to respiratory epithelial cells.[17]

The molecular basis for overexpression of epithelial ICAM-1 in asthma is uncertain, since IFN-γ levels derived from Th1 type T cells are expected to be low in this condition compared with viral infection.[18] Even when airway tissue levels of IFN-γ appear to be low, however, there may be overactivation of IFN-γ signal transduction, as evidenced by higher than normal levels of Stat1 activation.[19] Thus, airway epithelial cells may be programmed for constitutive ICAM-1 expression (mediated by Stat1-dependent pathways)[4,5,7] even in the apparent absence of viral infection or IFN-γ production.

The ability of cytokines to influence leukocyte-endothelial cell interactions and therefore to modulate leukocyte recruitment can be a powerful mechanism through which cytokines control inflammatory and immune responses. While up-regulation of specific adhesion pathways and the subsequent recruitment of select leukocyte subsets are critically important for the inflammatory reactions that protect us from infection, it is equally important for these to be reversed once the infection is eliminated. Tight control of neutrophil recruitment, in particular, is required, as neutrophils are potent reservoirs of degradative enzymes and reactive oxygen species, which can mediate significant tissue damage if uncontrolled.[20)]

Studies by Hallmann and coworkers[21)] have suggested that an alternative mechanism by which IFN-γ can inhibit neutrophil recruitment may be through modulation of endothelial cell adhesion molecule expression or function.

In conclusion, the ability of IFN-γ to control endothelial cell adhesion molecule expression and thus to regulate leukocyte recruitment is a powerful mechanism through which IFN-γ can direct immune and inflammatory reactions. Novel candidate therapeutics that target components of this process (cytokines, adhesion molecules, chemokines) have a promising future for treatment of autoimmune and inflammatory conditions in which overproduction of IFN-γ has pathologic consequences. In this regard, IFN-γ plus TNF-α or IL-1β can also synergistically affect Stat1-α/Group A Streptococcus interactions, as shown convincingly for ICAM-1. The current report indicates at least two mechanisms by which IFN-γ plus either TNF-α or IL-1β can activate similar promoter elements. Endogenous protein tyrosine kinase activity of oncoproteins, as well as constitutive activity of cytokine receptors or receptor-associated Janus kinases (Jaks), has been shown to drive Stat activation. Thus, abnormalities in the Jak-Stat pathway for cytokine signal transformation may be associated with disease due to abnormal cell growth, but no abnormality in this (or any other) pathway for cytokine signaling has yet been linked to the development of inflammatory disease.

These findings are identical to the profiles of Stat1 activation and Stat1-dependent gene expression triggered by IFN-γ signal transduction in isolated human airway epithelial cells and endobronchial explants, as well as in mouse models of airway inflammation.[4-7,14,19,22-24)] In all these cases, activation of the IFN-γ-receptor complex leads to Stat1 phosphorylation, dimerization, nuclear translocation and consequent binding to genetic targets, thereby enhancing gene transcription and expression. In particular, we found selective activity of epithelial Stat1 through a pathway that normally responds to production of the Th1 cytokine IFN-γ. This Stat1 signal may lead to overexpression from a subset of epithelial immune response genes, including ICAM-1, Stat1 and IFN regulatory Factor-1 (IRF-1) transcription factor,[4-7,19)] and to a condition of persistent immune activation. These correlations were less apparent in studies of endogenous gene activation and expression in primary cultured human airway epithelial cells.

Stat1 is functional (as assessed by its capacities for phosphorylation, nuclear translocation, activation of the ICAM-1 promoter, or induction of ICAM-1) in SAEC and LAEC. It can compete with endogenous Stat1 at the level of recruitment to the activated IFN-γ receptor.

The biologic context for the present work includes the finding that the IFN-γ-driven expression of airway epithelial ICAM-1 (as well as IRF-1) and regulated upon activation, normal T cell expressed and secreted (RANTES) is a major determinant of epithelial immune cell interaction.[8,14,22,23)] In addition, it appears that the activity of this IFN-γ-driven pathway (monitored by Stat1 activation and target gene expression) is increased during the immune response to respiratory viruses, and to the inflammatory response that is characteristic of asthma and lung transplant rejection.[19,25,26)] It is likely, therefore, that an IFN-γ-driven, Stat1-dependent subset of immune response genes (including ICAM-1, IRF-1, RANTES, and Stat1 itself) provides a molecular link between respiratory viral infection and the development of asthma.

The present results imply that naturally occurring or genetically engineered defects in the phosphorylation-dependent activation of Stat1 will provide the most enlightening insight into the role of Stat1 in mediating airway epithelial immunity and inflammation.

Conclusion

Cytokine responsiveness of SAEC and LAEC was evaluated to define the mechanism of lung allograft rejection, ICAM-1 expression on both LAEC and SAEC was significantly increased by IFN-γ stimulation alone and was synergistically enhanced by IFN-γ plus TNF-α or IL-1β stimulation. However, there was no difference in functional terms concerning cytokine responsiveness. Moreover, intranuclear displacement of Stat1 in SAEC by stimulation with IFN-γ plus TNF-α or IL-1β was recognized earlier, in comparison with that seen after IFN-γ

stimulation alone. Thus, the mechanisms and extent of cytokine responsiveness in SAEC, which were previously largely unknown, were revealed by this study, providing important information on aspects of chronic lung allograft rejection that have been recognized around the small airway.

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