Syk tyrosine kinase participates in β1-integrin signaling and inflammatory responses in airway epithelial cells

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Submitted 1 July 2004; accepted in final form 12 November 2004

Syk tyrosine kinase participates in β1-integrin signaling and inflammatory responses in airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 288: L497–L507, 2005. First published November 19, 2004; doi:10.1152/ajplung.00246.2004.—The protein tyrosine kinase Syk is widely expressed in hematopoietic cells and is involved in immunoreceptor signaling, cytokine signaling, and PI3K activity in breast cancer cells, and this is one possible mechanism for Syk’s effect on these cells (31). However, the function of Syk in these cells is poorly understood. Syk is expressed in breast epithelium, and loss of Syk is associated with malignancy in breast cancer cells, implicating the possible role of Syk in regulation of breast epithelial cell (EC) growth and differentiation (7). Syk inhibits PI3K activity in breast cancer cells, and this is one possible mechanism for Syk’s effect on these cells (31). However, the receptor(s) in these cells that initiate Syk signaling pathways are unknown. Furthermore, it is unclear whether other types of EC express Syk, and if so, what is the function(s) of Syk in these cells.

Airway EC express several receptors that enable recognition of soluble and cell-associated molecules in the microenvironment, including components of the extracellular matrix (ECM). Among EC receptors, integrins serve as important molecular sensors for dynamic changes in the extracellular environment. At least eight different members of the integrin family can be simultaneously expressed in airway EC, representing receptors for various ECM proteins such as fibronectin, collagen I, laminin-5, vitronectin, and tenascin (43). Integrin receptors mediate both inside-out and outside-in signaling, and in response to integrin receptor engagement, cells may undergo profound change in their functions (15). Cell proliferation, differentiation, adhesion, motility, and cytokine and chemokine production may all be modulated by integrin signaling. However, integrin-mediated signaling pathways in airway EC are poorly understood.

We hypothesized that Syk is expressed in the airway epithelium and that it participates in integrin signaling. In this study, we have shown that Syk is expressed in human bronchial EC and that it is involved in β1-integrin-mediated signaling. As Syk inhibition results in downregulation of proinflammatory molecules IL-6 and intercellular adhesion molecule-1 (ICAM-1), it emphasizes the role of Syk in inflammatory responses in the lung.

THE NONRECEPTOR PROTEIN TYROSINE KINASE Syk is widely expressed in hematopoietic cells. Syk contains two SH2 domains in the NH2-terminal region and a COOH-terminal kinase domain. The SH2 domains bind phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domain of immunoreceptors such as Fc receptors, the B cell receptor, and DAP12. In addition, multiple tyrosines present both in the kinase domain and interdomains, when phosphorylated, may serve as docking sites for other signaling proteins (13, 16, 47). Phosphorylated Syk kinase recruits and activates multiple downstream signaling molecules, including MAPK, phospholipase A, phospholipase C (PLC)γ, PKC, serine/threonine kinases, phosphatidylinositol 3-kinase (PI3K), and small GTPases (Rac1 and Cdc42) (8, 11, 25). Syk is critically involved in immunoreceptor signaling (8, 11, 19, 20) and participates in cytokine signaling (5, 40). Recent studies have also demonstrated Syk involvement in integrin-mediated signaling in hematopoietic cells (29, 34, 38, 53).

Although earlier studies focused on the role of Syk kinase in hematopoietic cells, it has recently become clear that Syk is expressed in some nonhematopoietic cells as well (7, 48, 52, 55). However, the function of Syk in these cells is poorly understood. Syk is expressed in breast epithelium, and loss of Syk is associated with malignancy in breast cancer cells, implicating the possible role of Syk in regulation of breast epithelial cell (EC) growth and differentiation (7). Syk inhibits PI3K activity in breast cancer cells, and this is one possible mechanism for Syk’s effect on these cells (31). However, the receptor(s) in these cells that initiate Syk signaling pathways are unknown. Furthermore, it is unclear whether other types of EC express Syk, and if so, what is the function(s) of Syk in these cells.

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L497
Cedarlane, Hornby, ON); MAb to CD18 or integrin (4B7R; Calbiochem, San Diego, CA) and 12G10 (Serotec, (ATCC), was cultured in RPMI 1640 with 1.5 mM-glutamine and gies), and 10% FBS. HT-29, human colorectal carcinoma cell line was cultured in minimum essential medium (Eagle’s) with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Life Technologies, Grand Island, NY) supplemented by L-glutamine (Sigma) and 5% FBS (Hyclone, Logan, UT).

BEAS-2B, human bronchial EC transformed by adenovirus 12-simian virus 40 (22), were acquired from American Type Culture Collection (ATCC; Rockville, MD) and cultured in bronchial EC growth medium (Clonetics) according to ATCC guidelines. The human pulmonary type II-like EC line A549 (ATCC) was cultured in Ham’s F-12K medium (Life Technologies) with 2 mM l-glutamine and 10% FBS. Calu-3, human lung adenocarcinoma cell line (ATCC), was cultured in minimum essential medium (Eagle’s) with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Life Technologies), and 10% FBS. HT-29, human colorectal carcinoma cell line (ATCC), was cultured in RPMI 1640 with 1.5 mM l-glutamine and 10% FBS.

**Immunohistochemistry analysis.** Human lung histopathological material including samples from adults and children (n = 5) was obtained from the University of Alberta Hospital, Edmonton, Alberta, Canada. Among the samples were two surgical specimens, representing visually normal lung tissue, and three samples from autopsy material were analyzed. The study was approved by the Health Research Ethics Board, University of Alberta.

For immunohistochemistry of rodent lung tissue, adult male Brown Norway rats (Harlan Sprague-Dawley, Indianapolis, IN) and C57Br/6 mice (Jackson Laboratories) were killed, and the lungs were inflated by instilling 10% buffered formalin, followed by fixation in the same solution for 24 h. The work was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with guidelines of the Canadian Council for Animal Care.

Sample block preparation, staining, and analyses were performed by the University of Alberta Hospital’s Department of Laboratory Medicine and Pathology. Briefly, following fixation, the lung tissue samples were embedded in paraffin and sectioned at 4 μm. Immunoperoxidase staining was performed using polyconal Ab to Syk. In parallel, slides were incubated with purified rabbit IgG instead of anti-Syk, used in the same concentration (isotype control). To further confirm the specificity of immunostaining, anti-Syk Ab was preincubated with a fivefold excess of blocking peptide (Santa Cruz Biotechnology) for 2 h at room temperature before the staining.

**Stimulation of cells by plating on fibronectin-coated surface or by β1-integrin antibody cross-linking.** Confluent PBEC or HS-24 cells were preincubated in serum-free culture medium for 1.5 h, removed using Ca2+- and Mg2+-free Hanks’ balanced salt solution (HBSS) with 1 mM EDTA, and washed and resuspended in serum-free medium.

Twelve-well culture plates were precoated with human plasma fibronectin (20 μg/ml; GIBCO and Invitrogen, Grand Island, NY), washed, and blocked with 2% BSA in PBS. Cells were distributed to the wells (0.25 × 104/well) and incubated at 37°C for different time periods. In some experiments, cells were preincubated with 100 μg/ml of RGD (H-Arg-Gly-Asp-OH) or GRADSP (H-Gly-Arg-Ala-Asp-Ser-Pro-OH) peptide (Calbiochem) for 30 min before being plated on a fibronectin-coated surface.

For stimulation with Abs, cells (1.5 × 105) were incubated in serum-free medium alone or medium containing 5 μg/ml of β1 MAb (4B7R) for 1 h at 4°C, washed, and incubated with 5 μg/ml of F(ab’)2 goat anti-mouse IgG and peroxidase-conjugated goat anti-mouse and anti-rabbit IgG, F(ab’); fragment specific (Jackson ImmunoResearch Laboratories, West Grove, PA); FITC- and PE-conjugated goat anti-mouse IgG (BD Biosciences); BODIPY goat anti-rabbit and anti-mouse IgG (heavy and light chains (H-L)); and rhodamine red X goat anti-rabbit IgG (H-L). 4’,6-Diamidino-2-phenylindole (DAPI) nucleic acid stain was from Molecular Probes (Eugene, OR). All other chemicals were from Sigma (Oakville, ON, Canada) unless otherwise indicated.

**Cell culture.** Cryopreserved primary normal human bronchial epithelial cells (PBEC) were purchased from Clonetics (Walkersville, MD) and grown in bronchial EC growth medium supplied by Clonetics according to the manufacturer’s instructions.

HS-24, a tumor cell line with properties of human bronchial EC (1, 32), was generously provided by Dr. E. Spiess (Heidelberg, Germany). The cells were grown in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented by L-glutamine (Sigma) and 5% FBS (Hyclone, Logan, UT).

RT-PCR. Total RNA from HS-24 cells was isolated with the QIAshredder and Qiagen RNeasy kit (Qiagen, Mississauga, ON, Canada). The RNA samples of every experiment were standardized to 18S-actin and Syk were amplified for 29 and 33 cycles, respectively, to stop the reaction in the log phase and ensure availability of all reagents. Primer sequences and other related information are summarized in Table 1.

Syk-small interfering RNA transfection. Small interfering (si)RNA targeted human Syk with base pairs of 364 –382 (5’-AACCTCATGAGGAAATGCGG-3’ (sense 5’-UUGGAGUAGUCCCUUAUAGAC-5’ (antisense 3’-GUU-3’ (antisense 3’-AACCTCATGAGGAAATGCGG-3’ (sense 5’-UUGGAGUAGUCCCUUAUAGAC-5’ (antisense 3’-GUU-3’ (antisense 3’-AACCTCATGAGGAAATGCGG-3’ (sense 5’-UUGGAGUAGUCCCUUAUAGAC-5’ (antisense 3’-GUU-3’ (antisense 3’-AACCTCATGAGGAAATGCGG-3’ (sense 5’-UUGGAGUAGUCCCUUAUAGAC-5’ (antisense 3’-GUU-3’ (antisense 3’-AACCTCATGAGGAAATGCGG-3’ (sense 5’-UUGGAGUAGUCCCUUAUAGAC-5’ (antisense 3’-GUU-3’ (antisense 3’-AACCTCATGAGGAAATGCGG-3’ (sense 5’-UUGGAGUAGUCCCUUAUAGAC-5’ (antisense 3’-GUU-3’ (antisense 3’-AACCTCATGAGGAAATGCGG-3’ (sense 5’-UUGGAGUAGUCCCUUAUAGAC-5’ (antisense 3’-GUU-
HS-24 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen Life Technologies, Burlington, ON, Canada) according to the manufacturer’s instructions. Briefly, 2 × 10⁵ cells/well in a 12-well plate were transfected with 3 μg of Syk-siRNA or control siRNA diluted in 100 μl of OPTI-MEM (GIBCO, Invitrogen) and mixed with 100 μl of OPTI-MEM containing 4 μl of Lipofectamine 2000. After a 4-h incubation of cells with siRNA-Lipofectamine 2000 complexes, medium was changed into a regular one, and cells were maintained for a total of 48 h. Harvested cells were used for RNA or protein extraction. In some experiments, cells were treated with 10 μM piceatannol (Calbiochem) or stimulated with 10 ng/ml of human recombinant tumor necrosis factor (TNF; Calbiochem) during the last 5 h of culture. The cells were resuspended in 100 μl of cytoseal (Richard-Allan Scientific, Kalamazoo, MI). Slides were examined using a Zeiss confocal microscope (LSM510) with a ×40 1.3 oil plan-Neofluar objective.

Immunofluorescent staining and LCM. Immunofluorescent staining was performed on coverslips precoated with 20 μg/ml of fibronectin or 0.01% poly-L-lysine. Cells adherent to coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. After blocking with PBS containing 3% BSA and 10% FBS, immunostaining with primary and then secondary Ab was performed. Rabbit IgG or mouse IgG1 were used as isotype controls. Finally, nuclear staining was observed in the basal cells (Fig. 1E). In the type 1 pneumocytes, both cytoplasmic and nuclear staining was observed (Fig. 1F). Type II pneumocytes were not readily seen in the examined lung specimens. The distribution and abundance of Syk staining were similar in bronchial and cartilageous airway epithelium.

In mouse (Fig. 1, G and H) and rat (not shown) lung, Syk staining was observed in airway epithelium of both small and large airways. However, the staining pattern was focal and of less intensity than in human lung. The staining was strongest in the apical part of cytoplasm of the ciliated columnar cells (Fig. 1H). Strong staining was also observed in smooth muscle in vessels and airways (Fig. 1H).

In all experiments, when polyclonal rabbit IgG (isotype control) rather than anti-Syk was used (Fig. 1, C and I) or when anti-Syk Ab was preincubated with the blocking peptide before applying to slides (not shown), immunoperoxidase reaction was negative, indicating that the immunostaining was Syk specific.

In addition to detection of Syk in airway EC in situ, Syk protein expression was also demonstrated in PBEC by Western blotting (Fig. 1J). Whereas the HS-24, bronchial cancer EC, and BEAS-2B adenovirus-transformed normal bronchial EC were Syk positive, the human type II pneumocyte cell line A549 and lung adenocarcinoma Calu-3 were negative (Table 2). Interestingly, Syk was also detected in the human colorectal carcinoma cell line HT-29, demonstrating that Syk expression in EC is not restricted to the airways (Table 2).

Cellular localization of Syk. LCM with polyclonal Ab showed abundant Syk expression in PBEC (Fig. 2A) and HS-24 cells (Fig. 2C). Although the known role of Syk as a nonreceptor tyrosine kinase may predict its cytoplasmic localization, we observed Syk in cytoplasmic, perinuclear, and nuclear areas in both types of human bronchial EC. Similar data were obtained using MAb to Syk (not shown).

With the use of either rabbit polyclonal IgG (Fig. 2B) or mouse IgG1 (not shown) or anti-Syk preincubated with the specific blocking peptide (Fig. 2D), virtually no fluorescence was detected.

Expression of integrins in bronchial EC. Syk expression in bronchial EC implicates its involvement in signal transduction in these cells. We postulated that Syk may be involved in integrin-mediated signaling. Indeed, β1-integrin (CD29) was detected by flow cytometry analysis in virtually 100% of both PBEC (Fig. 3A) and HS-24 cells (Fig. 3B), with higher expression on HS-24 (mean fluorescence units, 455.4 for HS-24 cells and 181.5 for PBEC). In the airway epithelium, β1-integrin can form heterodimers with several α-chains, particularly α5, α6, α5, and α6 (44). We observed expression of the α5-subunit (CD49e) on 85–95% of PBEC (Fig. 3A) and 70–80% of HS-24 cells (Fig. 3B; n = 3) indicating the presence of α5β1 heterodimer, the fibronectin receptor. In agreement with previous...
observations (44), β2-integrin (CD18) was absent from both bronchial EC types (data not shown).

**Cellular redistribution of Syk and β1-integrin following adhesion to fibronectin.** Because α5β1 is a fibronectin receptor, we used this ECM protein to engage β1-integrins. PBEC or HS-24 cells were plated on fibronectin-coated surfaces, followed by immunofluorescence staining for both Syk and β1-integrin and analysis using LCM. Thirty-minute adhesion to fibronectin induced visible spreading of both PBEC and HS-24 cells on coverslips (Fig. 4, A and C). By contrast, cells plated on poly-L-lysine-coated coverslips (30 min) were smaller and more rounded (Fig. 4, B and D).

After adhesion to fibronectin, Syk and β1-integrin in PBEC colocalized along the plasma membrane as thick linear structures (Fig. 4A), whereas in HS-24 cells, Syk and β1-integrin colocalized to large aggregates associated with the plasma membrane (Fig. 4C). After fibronectin treatment, β1-integrin localized to the plasma membrane as a thick layer in PBEC...
Table 2. Expression of Syk in human epithelial cell lines (Western blot analysis)

<table>
<thead>
<tr>
<th>Epithelial Cells</th>
<th>Syk Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary human bronchial epithelial cells</td>
<td>yes</td>
</tr>
<tr>
<td>HS-24, human bronchial squamous carcinoma cell line</td>
<td>yes</td>
</tr>
<tr>
<td>BEAS-2B, human bronchial epithelial cell, transformed by adenovirus 12-SV40</td>
<td>yes</td>
</tr>
<tr>
<td>A-549, human type II pneumocyte (cancer cell line)</td>
<td>no</td>
</tr>
<tr>
<td>Calu-3, human lung adenocarcinoma</td>
<td>no</td>
</tr>
<tr>
<td>HT-29, human colorectal carcinoma</td>
<td>yes</td>
</tr>
</tbody>
</table>

(Fig. 4A) and as large aggregates in HS-24 cells (Fig. 4C). Interestingly, following the plating of cells on fibronectin, β1-integrin was also visible in the nuclear region of both cell types (Fig. 4, A and C).

In PBEC or HS-24 cells plated on poly-L-lysine-coated coverslips and thus not activated via α5β1-integrin, Syk was homogeneously distributed in the cytoplasm with preferential localization to the nuclear and perinuclear area (Fig. 4, B and D). β1-Integrin in these cells was homogeneously localized to the plasma membrane as a thin layer (Fig. 4, B and D).

Cellular redistribution of Syk following stimulation of integrin receptors by antibody cross-linking. Integrin receptor ligation by ECM proteins results in integrin clustering and initiation of several signaling pathways (18). Integrin clustering can also be induced by Ab-mediated cross-linking of the receptors under nonadhesion conditions (27).

PBEC or HS-24 cells were stimulated by β1-integrin Ab cross-linking in nonadherent conditions for different times (1, 10, and 30 min) and then plated on poly-L-lysine-coated coverslips. LCM analysis showed redistribution of Syk induced by β1-integrin Ab cross-linking in both PBEC and HS-24 cells. Compared with control experiments, in which cells were incubated with the secondary reagent alone (Fig. 5A), or with anti-β2-integrin antibody instead of anti-β1-integrin (Fig. 5B), β1-integrin Ab cross-linking with secondary Ab induced visible changes in Syk expression (Fig. 5, C–E). Within 1 min following β1-integrin Ab cross-linking, cytoplasmic Syk expression in both cell types appeared less homogeneous (Fig. 5C) than in unstimulated cells. Ten minutes later, Syk was observed mainly on the plasma membrane (Fig. 5D), and in 30 min, it localized to both the plasma membrane and the perinuclear area (Fig. 5E). In HS-24 cells, in contrast to stimulation of cells by fibronectin (Fig. 4, C and D), Ab cross-linking was associated with more homogeneous distribution of Syk on the plasma membrane, without large clusters (Fig. 5, D and E, right). In PBEC, following 10 or 30 min of cross-linking with β1-integrin Ab, Syk distributions were patchy and associated with the cell surface and perinuclear region (30 min; Fig. 5, D and E, left).

β1-Integrin clustering results in activation of Syk. To test the hypothesis that Syk is involved in β1-integrin signaling, HS-24 cells were stimulated by specific antibody to β1-integrin, followed by immunoprecipitation of Syk and Western blot analyses of phosphorylated tyrosines in the immunoprecipitates. The results showed that this stimulation induced Syk phosphorylation on tyrosine, a direct indicator of its activation (Fig. 6A). The effect of antibody cross-linking was β1-integrin specific, since no Syk phosphorylation on tyrosine was detected when anti-β2-integrin MAb (of the same isotype) was used instead of anti-β1-integrin (not shown). In addition, HS-24 cells did not express Fcγ or Fcα receptors on the surface, as tested by flow cytometry using MAb to CD16, CD32, CD64, and CD89 (not shown), making it unlikely that Syk phosphorylation was due to Ab binding to Fc receptors.

To investigate whether Syk activation is also induced by cell stimulation with the natural β1-integrin ligand, HS-24 cells were plated on fibronectin-coated dishes for 15 min. Total lysates from cells adherent to fibronectin or from cells incubated in nonadherent conditions were examined for Syk phosphorylation on Tyr525/526 using Western blot analysis. As shown in Fig. 6B, Syk phosphorylation was greatly enhanced in cells adherent to fibronectin compared with nonadherent cells. To test whether Syk phosphorylation induced by fibronectin was specifically mediated by integrin receptors, cells were preincubated with the inhibitory (RGD) or control (GRADSP) peptide and then plated on fibronectin. The RGD
peptide inhibited Syk phosphorylation, but the control peptide did not (Fig. 6B).

Inhibition of Syk downregulates TNF-dependent ICAM-1 expression and IL-6 release. Because Syk is able to activate some proinflammatory signaling pathways in hematopoietic cells (11), it is possible that it can also mediate inflammatory responses in airway epithelium. To assess Syk involvement in regulation of inflammatory responses by EC, we used siRNA to suppress its expression. We transiently transfected HS-24 cells with Syk siRNA or control siRNA and then examined cell

Fig. 3. PBEC and the HS-24 cell line express β₁-integrin (CD29) and α₅-integrin (CD49e). Flow cytometric analysis was conducted on PBEC (A) and HS-24 cells (B) using immunostaining with CD29 (top) or CD49e MAb (bottom) (shaded histograms) or isotype control (mouse IgG1, open histograms). The ordinate displays cell counts, and the abscissa displays the fluorescence intensity in arbitrary units. Numbers represent mean fluorescence units. The results represent data of 3 independent experiments.

Fig. 4. β₁-Integrin receptor engagement via adhesion to fibronectin mediates cellular re-distribution of Syk and β₁-integrin in bronchial epithelial cells. PBEC (A and B) or the HS-24 cell line (C and D) were plated on fibronectin (A and C) or poly-L-lysine (B and D)-coated glass coverslips for 30 min, fixed, and stained with rabbit polyclonal Ab to Syk (i, green) and with MAb to CD29 (ii, red). Cells were counterstained with DAPI nuclear acid stain (i-iii and v, blue). The subcellular distribution of Syk and β₁-integrin was analyzed by confocal microscopy. The corresponding DIC images of the cells are also shown (iv). Colocalization of Syk and β₁-integrin is indicated by yellow staining in the merged images (arrows, A-iii, C-iii). Arrowheads indicate nuclear localization. Scale bars represent 5 µm. The images represent the results of 5 independent experiments.
expression both in resting cells (plated on poly-L-lysine) and cells adherent to fibronectin (Fig. 7C). Interestingly, cells adherent to fibronectin showed higher expression of ICAM-1 following stimulation with TNF compared with cells adherent to poly-L-lysine ($P < 0.05$). Transfection with Syk siRNA downregulated TNF-induced ICAM-1 expression in fibronectin-plated HS-24 cells ($P < 0.005$) but had no significant effect on ICAM-1 in poly-L-lysine-plated cells. Neither did control siRNA. Overnight treatment with the pharmacological Syk inhibitor piceatannol (10 μM) caused significant downregulation of ICAM-1 in TNF-stimulated cells both in poly-L-lysine- and fibronectin-adherent conditions (Fig. 7C). As determined by trypan blue dye exclusion, the treatment of cells with siRNA or piceatannol had no significant effect on viability (in all experiments, viability was $> 96\%$).

Although IL-6 release by HS-24 cells without TNF stimulation was minimal, there was a trend to higher IL-6 levels in culture supernatants of cells adherent to fibronectin (Fig. 7D). As expected, a great elevation of IL-6 levels was observed following TNF stimulation in both culture conditions, with higher levels in fibronectin-adherent cells ($P < 0.05$). Syk siRNA treatment caused downregulation of IL-6 release (55–58%) that reached statistical significance in fibronectin-adherent culture ($P < 0.05$). Piceatannol almost completely inhibited TNF-induced IL-6 release (Fig. 7D).

Thus, inhibition of Syk downregulated TNF-induced expression of both ICAM-1 and IL-6, hallmarks of inflammatory responses in the airway epithelium. The effect was significant in cells adherent to fibronectin, implicating that Syk involvement in these proinflammatory events is at least partly $\beta_1$-integrin dependent.

**DISCUSSION**

The airway epithelium is responsive to various environmental factors and is an active participant in inflammatory and immune responses. EC interact with ECM and cells in the microenvironment and respond to cytokines, chemokines, hormones, growth factors, and inflammatory mediators. EC can release a wide range of biologically active molecules that act as pivotal regulators in the respiratory tract. It has been postulated that EC play a crucial role in airway inflammation, smooth muscle hyperresponsiveness, and remodeling, particularly in asthma (17). However, intracellular signaling pathways involved in regulation of EC are not completely understood.

Using several techniques, we show for the first time that the tyrosine kinase Syk is expressed in primary human bronchial EC and cell lines including the bronchial squamous carcinoma HS-24 and adenovirus-transformed BEAS-2B. In addition, we demonstrated Syk expression in bronchial epithelium in situ in humans, mice, and rats. Interestingly, several other respiratory EC lines tested, namely A-549, a human type II pneumocyte, and Calu-3, a human lung adenocarcinoma, were Syk negative.

Syk was originally identified in hematopoietic cells and involved in immunoreceptor signaling (8, 11, 19, 47). Recent studies by us (49) and others (48, 52, 55) have detected Syk expression in some nonhematopoietic cells, including breast epithelium and breast cancer cells (7). These observations raise the interesting possibility that Syk may be stimulated in the absence of any immunoreceptors known to recruit Syk via the...
"classic" interaction of phosphorylated ITAMs with Syk SH2 domains.

Our observations of Syk expression in bronchial EC pose two significant questions. First, which receptor engagement leads to Syk activation and signaling in the epithelium, and second, what is the functional role of Syk in these cells? We hypothesized that in airway EC, Syk mediates integrin signaling. Indeed, Syk was found to be involved in β1-, β2-, and β3-integrin signaling in hematopoietic cells (29, 34, 38, 53). Some surprising molecular characteristics of Syk interaction with integrins were detected: interaction of Syk with the β3-integrin involved SH2 domains but did not involve phosphorylation of the integrin cytoplasmic domain (51).

In airway EC, eight different integrin αβ heterodimers mediating cell-cell and cell-ECM interactions are expressed (reviewed by Ref. 43). Interactions of airway EC integrins with the growth factor TGF-β, as well as with matrix metalloproteinase-9, involved in inflammatory responses, tumorigenesis, and pulmonary fibrosis, have been postulated (12, 42). The integrin α5β1 can activate NF-κB (21), and the integrin α3β1 can modulate cytokine responses by lung EC (30). However, integrin-mediated signaling in airway epithelium is poorly understood.

Few published reports on molecular interactions between Syk and members of the integrin family are restricted to hematopoietic cells. Syk coimmunoprecipitated with β2 or αIIbβ3 following adhesion of neutrophils, macrophages, or platelets to ECM proteins (34, 36, 53, 54). Syk and β2-integrin colocalized at edges of activated neutrophils (34). However, apart from studies of Syk activation following β1-integrin engagement in monocytes and platelets (23, 29, 38), molecular interactions of Syk with β1-integrin have not been investigated.

We have provided several lines of evidence that Syk participates in β1-integrin signaling in bronchial EC. We focused on β1-integrin since it is highly expressed in both PBEC and HS-24 cells. In addition, the α5β1 subunit in the heterodimer α5β1 (fibronectin receptor) was detected. To stimulate β1-integrin, PBEC or HS-24 cells were plated on fibronectin-coated surfaces or stimulated via cross-linking of receptors with β1-integrin Ab in nonadherent conditions. Integrin receptor clustering by a specific ligand or Ab cross-linking leads to formation of focal adhesion complexes (10, 15, 18). Using LCM, we directly visualized large aggregates along the plasma membrane after plating cells on fibronectin, where Syk and β1-integrin colocalized. These structures were morphologically similar to previously described focal adhesions (9, 35, 41). When β1-integrins were clustered with Ab, dramatic cellular redistribution of both Syk and β1-integrin was also observed; however, the pattern was different. In nonadherent conditions, we did not see large clusters, but Syk and β1-integrin were rather homogeneously distributed along the plasma membrane. Previous observations demonstrated that integrin stimulation in adherent conditions resulted in more profound molecular changes compared with Ab-mediated receptor cross-linking, probably due to more complete cytoskeleton engagement (29).
Cellular redistribution of Syk and β1-integrin occurred in both PBEC and HS-24 cells, demonstrating that this phenomenon was common for both cell types.

Colocalization of Syk with β1-integrin occurred following integrin engagement but did not occur in resting cells. Thus no colocalization was detected when cells were plated on poly-L-lysine-coated surfaces, a condition known to inhibit integrin clustering (4). This indicates that during integrin clustering, Syk associates with β1-integrin, suggesting a functional interaction. Indeed, tyrosine phosphorylation of Syk occurred as a result of β1-integrin engagement. Phosphorylation of Syk on tyrosine is a direct sign of Syk activation (56), and it was previously observed following β1- and β2-integrin engagement in monocytes (29) and neutrophils (34).

Finally, following integrin cross-linking, cellular redistribution of both Syk and β1-integrin occurred. Although believed to be a cytoplasmic protein, Syk was also clearly detected by confocal analysis in the perinuclear and nuclear area in cultured bronchial EC as well as by immunohistochemistry in the nuclear area in the airway epithelium and type I pneumocytes in situ. Our data corroborate recently published data on Syk detected in the nuclear fraction of breast EC (50), suggesting that in EC, Syk localizes to the nucleus and may interact with nuclear components.

In the current model of integrin signaling, large multimolecular complexes are formed that involve cytoskeleton proteins, focal adhesion kinase, other kinases, and adaptor proteins (6, 10, 15, 28). The role of Syk in these complexes and how essential Syk is for the downstream signaling from integrin receptors in airway EC need to be established. Given that Syk is capable of inducing phosphorylation of many signaling proteins, e.g., PLC, MAPK cascade, and PI3K (8, 11, 25), its role in regulation of integrin-dependent EC functions may be important. Various aspects of cell behavior such as proliferation, survival, differentiation, establishing cell polarity, and production of cytokines, chemokines, metalloproteases, and ion and mucus secretion are possibly regulated by Syk signaling.

We hypothesized that in airway EC, Syk regulates inflammatory responses following its recruitment into β1-integrin-mediated signaling cascade. To investigate Syk involvement in inflammatory responses, we used siRNA and analyzed expression of the adhesion molecule ICAM-1 and cytokine IL-6, both proinflammatory factors, produced by activated respiratory epithelium (37). Because the expression of both ICAM-1 and IL-6 is regulated by the transcription factor NF-κB (2, 39), we used one of the potent NF-κB activators, TNF, to stimulate ICAM-1 and IL-6 in HS-24 cells (14). Specific inhibition of Syk with siRNA downregulated both ICAM-1 and IL-6 induced by TNF. These observations directly implicate Syk in proinflammatory responses in lung EC. Compared with Syk siRNA, piceatannol had a stronger effect and caused complete inhibition of TNF-induced ICAM-1 and IL-6 expression in EC. A possible explanation is that this inhibitor is not entirely Syk specific and can suppress other signaling molecules as well. Indeed, it was recently demonstrated that piceatannol directly inhibits TNF-induced NF-κB activation and NF-κB-mediated gene expression through suppression of IKK and p65 phosphorylation, and this effect is Syk independent (3).

It is tempting to speculate that Syk is involved in regulation of ICAM-1 and IL-6 expression at the transcriptional level, particularly via its direct or indirect interaction with NF-κB. Although previous studies demonstrated that in a monocyte cell line, activation of both Syk and NF-κB occurred upon β1-integrin ligation, direct evidence of Syk interaction with any component of NF-κB complex is missing (29). To the best of our knowledge, there is no published data dissecting molecular mechanisms of possible interaction of Syk with the NF-κB signaling pathway induced via the TNF receptor superfamily. Although a recent study showed that Syk induces tyrosine phosphorylation of IκBα, leading to the nuclear translocation of p65 in myeloid cells activated by H2O2, this involved a different pathway of NF-κB activation (46). Mechanisms of Syk involvement in regulation of ICAM-1 and IL-6 expression may also act at the posttranscriptional level, given that Syk activates multiple downstream signaling pathways (8, 25).

Our observations that EC adherent to fibronectin respond to TNF stimulation with higher ICAM-1 and IL-6 than cells plated on poly-L-lysine corroborate previous observations that in different cell types, integrin signaling can regulate NF-κB activation (24, 26, 29, 38). The currently accepted concept postulates that integrins provide a costimulatory signal during cell activation via other receptors, e.g., cytokine or growth factor receptors (24, 33).

Suppression of Syk by siRNA caused only partial inhibition of ICAM-1 and IL-6 expression, and the inhibitory effect was stronger in cells adherent to fibronectin. It is possible that the proinflammatory effect of Syk in EC is at least partly β1-integrin dependent, or in other words, Syk is not significantly involved in the regulation of ICAM-1 and IL-6 in the absence of β1-integrin stimulation. These observations speak in favor of cross talk between integrin and TNF receptor signaling in airway EC, potentially involved in regulation of inflammatory responses in the lung.

At present, Syk is considered an attractive target for anti-inflammatory therapy. Indeed, we have established that specific inhibition of Syk in the lung using aerosolized antisense significantly decreased inflammatory responses in the airways (44, 45). Although we initially identified alveolar macrophages as cellular targets of local Syk antisense treatment (44), inhibition of Syk expression in the airway EC may represent another important mechanism of anti-inflammatory action.

ACKNOWLEDGMENTS

We are grateful to Dr. Eberhard Spiess (Heidelberg, Germany) for providing the HS-24 cell line, Rene Dery (Univ. of Alberta, Edmonton, AB, Canada) for skillful technical help, and personnel of Cell Imaging Facilities, Cross Cancer Institute (Edmonton), Dr. Xuejun Sun, and Gerry Barron for help with confocal microscopy.

GRANTS

This work was supported by a Canadian Institutes for Health Research Grant, National Heart, Lung, and Blood Institute Grants HL-27068 and HL-69498, and postdoctoral fellowships from the Canadian Society of Allergy and Clinical Immunology/Merck Frosst and from the Alberta Heritage Foundation for Medical Research.

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