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The fibroblasts stimulated by cytokines released the chemokine and recruited the infiltrating cells, including eosinophils, that play a key role in the pathogenesis of airway disease. The fibroblasts released the chemokine RANTES or GM-CSF and recruited the infiltrating cells, including eosinophils (1). High amounts of RANTES were found in the nasal polyp specimens (2). Cultured nasal fibroblasts expressed RANTES mRNA and secreted RANTES protein (3). We established the human fibroblast lines showing high Syk expression and the lines showing low Syk expression from pieces of nasal polyp (4), although Syk is widely expressed and plays an important role in intracellular signal transduction in hemopoietic cells, B cells (5), mast cells (6), platelets (7), eosinophils (8), and so on.

RANTES production requires the transcription factor NF-κB and the activation of mitogen-activated protein kinases (MAPKs); Refs. 9–13. IL-1 induces the interaction of TNFR-associated factor 6 (TRAF6) with IL-1R-associated kinase (IRAK), which is recruited rapidly to the IL-1R after IL-1 induction, whereas TRAF2 participates in TNF-α signaling. In the present study, we found that Syk played a different role in IL-1- and TNF-α-induced chemokine production through a signaling complex involving Syk and TRAF6. Overexpression of wild-type Syk by gene transfer enhanced RANTES production from nasal fibroblasts stimulated with IL-1. The decrease of Syk expression by the administration of Syk antisense inhibited RANTES production in response to IL-1. However, the change of Syk expression did not affect RANTES production by TNF-α stimulation. We concluded that Syk is required for the IL-1-induced chemokine production through the association with TRAF-6 in fibroblasts of nasal polyps. The Journal of Immunology, 2001, 167: 283–288.

Fibroblasts, a rich source of chemokines, interact with eosinophils and play a key role in the pathogenesis of airway disease. The fibroblasts released the chemokine RANTES or GM-CSF and recruited the infiltrating cells, including eosinophils (1). High amounts of RANTES were found in the nasal polyp specimens (2). Cultured nasal fibroblasts expressed RANTES mRNA and secreted RANTES protein (3). We established the human fibroblast lines showing high Syk expression and the lines showing low Syk expression from pieces of nasal polyp (4), although Syk is widely expressed and plays an important role in intracellular signal transduction in hemopoietic cells, B cells (5), mast cells (6), platelets (7), eosinophils (8), and so on.

RANTES production requires the transcription factor NF-κB and the activation of mitogen-activated protein kinases (MAPKs); Refs. 9–13. IL-1 induces the interaction of TNFR-associated factor 6 (TRAF6) with IL-1R-associated kinase (IRAK), which is recruited rapidly to the IL-1R after IL-1 induction, whereas TRAF2 participates in TNF-α signaling (14, 15). Recently, Wong et al. (16) demonstrated a signaling complex involving tyrosine kinase c-Src and TRAF6. Additionally, Li et al. (17) revealed that tyrosine kinase inhibitor substantially alleviated the cytokine gene expression and decreased the level and activity of IRAK.

In the present study, we examined whether Syk is involved in the RANTES production by IL-1 and TNF-α via TRAFs. We also looked at the phosphorylation state of c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), and p38 MAPK in response to IL-1 when Syk expression was artificially changed by oligonucleotides and vectors. Further, IL-1-induced IκB degradation was investigated as a reporter of NF-κB-signaling, a transcription factor essential for RANTES expression (18, 19).

Materials and Methods

Reagents

Anti-Syk Ab, anti-c-Src Ab, anti-TRAF6 Ab, anti-TRAF 2 Ab, anti-JNK Ab, anti-phosphorylated JNK Ab, anti-p38 Ab, anti-phosphorylated p38 Ab, anti-ERK Ab, and anti-phosphorylated ERK Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IL-1β was obtained from Cistron Biotechnology (Pine Brook, NJ). Recombinant human TNF-α and the ELISA kits for RANTES were purchased from Biosource (Camarillo, CA). The adenovirus expression vector kit was purchased from Takara Biomedicals (Tokyo, Japan).

Cell preparation

Nasal polyps were obtained during surgery from patients with chronic parasanal rhinosinusitis. Chronic rhinosinusitis was strictly diagnosed via endoscopic findings, parasanal sinus x-ray tomography, clinical history, and symptoms by a specialist of otorhinolaryngology. Nasal polyps were fluid-filled sacks formed from the upper part of the nasal cavity.

Human nasal fibroblast lines were grown from small pieces of nasal polyp and regular passages (20). Fibroblasts were used at passage numbers 4–10. No contamination of epithelial cells was confirmed by immunohistochemical examination with cytokeratin marker. There was wide variation in the expression of Syk by nasal fibroblast lines as described previously (4). Before starting the experiments, Western blot analysis was performed to be determined Syk expression in fibroblast cell line. In this study, we used the human fibroblast line with the highest Syk expression and the line with the lowest Syk expression in our library. We could not find any difference in growth characteristics or morphology between fibroblast line with the highest and that with the lowest Syk expression.

Cell stimulation and chemokine assay

The fibroblasts were stimulated by IL-1β and TNF-α in RPMI 1640 medium supplemented with 10% FCS and in humidified atmosphere of 10%
CO₂ in air at 37°C for 24 h. The supernatants were harvested and stored at –80°C. Amounts of RANTES in the cell culture supernatant were measured with an ELISA kit. Measurements were performed according to the manufacturers’ protocol.

Coimmunoprecipitation

Cells were collected by centrifugation and lysed in 1 ml of immunoprecipitation buffer (1% digitonin, 0.15 M NaCl, 5 mM η, 100 mM Na₂VO₄, 10 mg/ml leupeptin, 1 mM PMSF, and 50 mM Tris-HCl, pH 7.5). The lysate was clarified and incubated with excess protein A-Sepharose 4B (50% slurry). The cleared sample was immunoprecipitated with Abs and protein A-Sepharose 4B at 4°C. The immune complexes were processed for immunoblot as described above.

Gel electrophoresis and Western blots

The fibroblasts or immunoprecipitants were boiled with electrophoresis sample buffer for 3 min and separated by SDS-PAGE. The separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes at the same protein concentration per lane. The membranes were blocked at room temperature for 1 h in pH 7.4 PBS with 10% BSA and were probed with Abs as described previously (4).

Preparation of recombinant adenoviruses

An adenovirus vector encoding Syk was constructed with the use of an adenovirus expression kit (Takara). A 2.7-kb cDNA fragment containing the entire coding sequence of Syk was ligated into the pXCAwt cosmid vector, which contains the modified chicken β-actin promoter with cytomegalovirus-IE enhancer (CAG promoter). Then, the recombinant adenovirus pXCAwt-Syk was prepared by homologous recombination of the expression cosmid cassette and parental viral genome and amplified to achieve a stock with a titer of ~10⁷ PFU/ml. A control recombinant adenovirus pXCAwt-LacZ was used. Fibroblasts were equally infected with 10 μM phosphorothionated Syk antisense oligodeoxynucleotides for 6 h inhibited the expression of Syk protein, whereas the exposure of Syk sense oligodeoxynucleotides did not change Syk protein levels. We examined c-Src expression in these fibroblasts with anti-c-Src Ab as a control. There were no differences in the levels of c-Src expression.

IL-1-dependent association of Syk with TRAF6

To examine whether Syk plays a role in the RANTES production, we artificially regulated the Syk expression in the fibroblast line with antisense oligodeoxynucleotides to Syk. As shown in Fig. 1B, human nasal fibroblasts exposed to 10 μM phosphorothionated Syk antisense oligodeoxynucleotides for 6 h inhibited the expression of Syk protein, whereas the exposure of Syk sense oligodeoxynucleotides did not change Syk protein levels. We examined c-Src expression in these fibroblasts with anti-c-Src Ab as a control. There were no differences in the levels of c-Src expression.

IL-1 caused the association of Syk with TRAF-6

We obtained the human fibroblast line showing high Syk expression. Production of RANTES by the fibroblasts was measured by ELISA. As shown in Fig. 1A, the levels of RANTES production by the fibroblasts in the presence of IL-1β (1 ng/ml) or TNF-α (1 ng/ml) was significantly elevated compared with those in the absence of IL-1 or TNF-α (p < 0.01).

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IL-1 failed to induce RANTES production from fibroblasts that did not exhibit Syk protein (Fig. 1A). Sense oligodeoxynucleotides did not alter IL-1-induced RANTES production. There was a significant difference in IL-1-induced RANTES production between fibroblasts treated with Syk antisense oligodeoxynucleotides and those with sense oligodeoxynucleotides (p < 0.01).

In contrast, there was no difference in TNF-α-induced RANTES production between fibroblasts treated with Syk antisense oligodeoxynucleotides and those with sense oligodeoxynucleotides (Fig. 1A).

Interestingly, the role of Syk for IL-1 signaling might be different from that of TNF-α signaling in RANTES production.

Results

Syk antisense oligodeoxynucleotides attenuated IL-1-induced RANTES production from human nasal fibroblasts

We obtained the human fibroblast line showing high Syk expression. Production of RANTES by the fibroblasts was measured by ELISA. As shown in Fig. 1A, the levels of RANTES production by the fibroblasts in the presence of IL-1β (1 ng/ml) or TNF-α (1 ng/ml) was significantly elevated compared with those in the absence of IL-1 or TNF-α (p < 0.01).

To examine whether Syk plays a role in the RANTES production, we artificially regulated the Syk expression in the fibroblast line with antisense oligodeoxynucleotides to Syk. As shown in Fig. 1B, human nasal fibroblasts exposed to 10 μM phosphorothionated Syk antisense oligodeoxynucleotides for 6 h inhibited the expression of Syk protein, whereas the exposure of Syk sense oligodeoxynucleotides did not change Syk protein levels. We examined c-Src expression in these fibroblasts with anti-c-Src Ab as a control. There were no differences in the levels of c-Src expression.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test and Wilcoxon signed-ranks test to assess the differences in RANTES production levels. Macintosh computers (Apple Computer, Cupertino, CA) with Statview software (Abacus Concepts, Berkeley, CA) were used for all statistical analyses.

FIGURE 1. Syk antisense oligodeoxynucleotides attenuated IL-1-induced RANTES production from human nasal fibroblasts. A, Nasal fibroblasts with high Syk expression (1 × 10⁷ PFU/ml) were cultured in the presence of 10 μM Syk antisense, 10 μM Syk sense, or in the absence of oligodeoxynucleotide for 6 h and then stimulated with IL-1β (1 ng/ml) or TNF-α (1 ng/ml). Supernatants were harvested for analysis of RANTES secretion by ELISA 24 h after the stimulation. The pretreatment of Syk antisense-inhibited RANTES production by fibroblasts stimulated with IL-1β (*, p < 0.01), but did not inhibit those stimulated with TNF-α. B, Nasal fibroblasts were transfected with Syk antisense, Syk sense, or no oligodeoxynucleotide. Harvested proteins (30 μg) from cultured cells were reacted with anti-Syk Ab and anti-c-Src Ab and then processed for immunoblotting. The arrows indicate the positions of Syk and c-Src.
associations after cell lysis (21). It has been determined that endogenous Syk, c-Src, and TRAF6 form a signaling complex in nasal fibroblasts after stimulation of IL-1.

As shown in Fig. 2A, immunoblot analysis revealed the existence of Syk in the immune complex precipitated by anti-TRAF6 Ab. IL-1 stimulation substantially increased the association of TRAF6 with Syk in nasal fibroblasts. Also, we found the existence of c-Src in the immune complex precipitated by anti-TRAF6 Ab after the stimulation of IL-1. In contrast, TNF-α stimulation caused no association of TRAF6 with Syk or with c-Src (Fig. 2A). When samples were immunoprecipitated with TRAF6 or TRAF2 and blotted with corresponding TRAF6 or TRAF2, respectively, there were no differences in the levels of TRAF6 and TRAF2 immunoprecipitates before and after stimulation. Also, we found no association of TRAF6 with Syk in the fibroblasts after TNF-α stimulation (data not shown).

Fig. 2B proved the existence of endogenous Syk, c-Src, TRAF6, and TRAF2 in the fibroblasts. No differences were found in the expression levels of these molecules with the samples before immunoprecipitation.

The effect of reduced Syk expression on the phosphorylation of JNK, p38, and ERK

Because IL-1 was reported to cause the activation of JNK (14, 15), we investigated IL-1-induced phosphorylation of JNK, ERK, and p38 MAPK. To analyze whether Syk plays some roles in regulating the phosphorylation of these molecules in response to IL-1, the fibroblasts were exposed to Syk sense or Syk antisense oligodeoxynucleotides, and then treated with IL-1 for 10 min. As shown in Fig. 3, cell lysates were subjected to immunoblotting with anti-phosphorylated JNK Ab, anti-phosphorylated p38 Ab, and anti-phosphorylated ERK Ab.

JNK was phosphorylated and activated after treatment with IL-1 for 10 min because the anti-phosphorylated JNK Ab reacted with active JNK1 phosphorylated on threonine 183 and tyrosine 185 (Fig. 3A, top; Ref. 4). JNK1 phosphorylation was almost maximal within 10 min and remained elevated throughout the 30-min incubation of IL-1 (data not shown). Phosphorylation of p38 also occurred by treatment with IL-1, with the anti-phosphorylated p38 Ab reacting with active p38 phosphorylated on tyrosine-182 (Fig. 3B, top).

The treatment with Syk sense oligodeoxynucleotides had no effect on the JNK and p38 phosphorylation induced by IL-1. However, IL-1 failed to induce JNK phosphorylation of the fibroblasts treated with Syk antisense oligodeoxynucleotides (Fig. 3A). Syk antisense oligodeoxynucleotides attenuated p38 phosphorylation induced by IL-1 (Fig. 3B). The semiquantitative densitometric analysis in p38 phosphorylation showed that the value in the Syk antisense-treated fibroblast was half of that in the Syk sense-treated fibroblast (0.38 vs 0.77).

In contrast, ERK was constantly phosphorylated on tyrosine 204 before the stimulation in the fibroblast lines and was not changed by stimulation of IL-1 (Fig. 3C). Syk antisense oligodeoxynucleotides did not affect the phosphorylation of ERK in the fibroblasts.

Wild-type Syk vector transfection magnified IL-1-induced RANTES production by nasal fibroblasts

We also established the human fibroblast line showing low Syk expression and transfected expression vector from wild-type Syk into the fibroblasts. As shown in Fig. 4A, there was no difference in the spontaneous RANTES production in the absence of IL-1 or TNF-α among the fibroblasts transfected with the different vectors.
Although TNF-α caused the high levels of RANTES production from the fibroblasts showing low Syk expression, IL-1 induced small amounts of RANTES production from the same fibroblasts. However, the fibroblasts transfected with the wild-type Syk vector produced significantly higher levels of RANTES after stimulation with IL-1 than those transfected with the control vector did ($p < 0.01$). Wild-type Syk vector transfection did not affect the TNF-α-induced RANTES levels from fibroblasts transfected with wild-type Syk vector and those from fibroblasts transfected with control vector. No difference was found in the morphology and cell growth among four fibroblasts even after the transfection of virus vector (data not shown).

The transfection of wild-type Syk vector enhanced the expression of Syk on day 2, whereas the control vector did not do so (Fig. 4B). We examined c-Src expression with anti-c-Src Ab in these fibroblasts transfected with vectors. There were no differences in the levels of c-Src expression, as shown in Fig. 4B.

The effect of increased Syk expression on the phosphorylation of JNK, p38, and ERK

After the fibroblasts showing low Syk expression were transfected with wild-type Syk vector or control vector, we investigated IL-1-induced phosphorylation of JNK, p38 MAPK, and ERK. The fibroblasts were transfected with wild-type Syk vector or control vector and then treated with IL-1 for 10 min. The cell lysates were subjected to immunoblotting with anti-phosphorylated JNK Ab, anti-phosphorylated p38 Ab, and anti-phosphorylated ERK Ab (Fig. 5).

The phosphorylation of JNK after IL-1 stimulation occurred more in the fibroblasts transfected with wild-type Syk vector than in the fibroblasts transfected with control vector (Fig. 5A, top). The transfection with wild-type Syk vector also amplified the p38-phosphorylation of the fibroblasts after stimulation with IL-1 for 10 min from 0.32 to 0.72 by semiquantitative densitometric analysis (Fig. 5B, top). However, ERK was phosphorylated without the stimulation in the fibroblasts transfected with wild-type Syk vector or control vector (Fig. 5C, top). Wild-type Syk vector did not affect ERK phosphorylation after IL-1 stimulation.

The effect of increased Syk expression on the phosphorylation of JNK, p38, and ERK

After the fibroblasts showing low Syk expression were transfected with wild-type Syk vector or control vector or no vector into the fibroblasts with low Syk expression for 48 h, the cells were incubated in the presence or absence of IL-1β (1 ng/ml) or TNF-α (1 ng/ml), and supernatants were harvested for analysis of RANTES secretion by ELISA. IL-1-induced RANTES levels from fibroblasts transfected with wild-type Syk vector were significantly higher than those from fibroblasts transfected with control vector ($p < 0.01$), whereas there was no difference between TNF-α-induced RANTES levels from fibroblasts transfected with wild-type Syk vector and those from fibroblasts transfected with control vector. B, Nasal fibroblasts with low Syk expression are used in this experiment. The prepared proteins (30 μg) were processed and reacted with anti-Syk Ab or anti-c-Src Ab. Anti-Syk immunoblotting showed the increased Syk expression in the fibroblasts transfected with wild-type Syk vector but did not in those transfected with control vector.

**FIGURE 4.** Wild-type Syk vector transfection magnified IL-1-induced RANTES production by nasal fibroblasts. A, After the transfection with wild-type Syk vector or control vector or no vector into the fibroblasts with low Syk expression for 48 h, the cells were incubated in the presence or absence of IL-1β (1 ng/ml) or TNF-α (1 ng/ml), and supernatants were harvested for analysis of RANTES secretion by ELISA. IL-1-induced RANTES levels from fibroblasts transfected with wild-type Syk vector were significantly higher than those from fibroblasts transfected with control vector ($*, p < 0.01$), whereas there was no difference between TNF-α-induced RANTES levels from fibroblasts transfected with wild-type Syk vector and those from fibroblasts transfected with control vector. B, Nasal fibroblasts with low Syk expression are used in this experiment. The prepared proteins (30 μg) were processed and reacted with anti-Syk Ab or anti-c-Src Ab. Anti-Syk immunoblotting showed the increased Syk expression in the fibroblasts transfected with wild-type Syk vector but did not in those transfected with control vector.

**FIGURE 5.** The effect of increased Syk expression on the phosphorylation of JNK, p38, and ERK. Nasal fibroblasts with low Syk expression were transfected with control vector (lanes 1 and 2) or wild-type Syk vector (lanes 3 and 4) for 48 h and then stimulated without IL-1β (lanes 1 and 3) or with 1 ng/ml IL-1β (lanes 2 and 4) for 10 min. The same amount of lysate (30 μg) was applied to each lane and blotted with anti-phosphorylated JNK Ab, anti-phosphorylated p38 Ab, and anti-phosphorylated ERK Ab (Fig. 5).
The effect of increased Syk expression on the degradation of IκB

Because IL-1 was reported to cause the activation of NF-κB (17), we investigated the effects of Syk expression on the degradation of IκB-α, which regulates NF-κB (Fig. 6). The fibroblasts showing low Syk expression were transfected with wild-type Syk vector or control vector and then stimulated with IL-1. IL-1 induced the degradation of IκB-α in the fibroblasts transfected with wild-type Syk vector, although it did not induce any degradation IκB-α in those transfected with control vector, suggesting that IL-1 induced NF-κB activation in the fibroblast showing high Syk expression.

**Discussion**

Although Syk expression was usually found in hemopoietic cells, mouse embryonic fibroblasts and rat hepatocytes recently were reported to express Syk (22, 23). We found that some human fibroblast lines highly expressed Syk protein (4). In this study, IL-1 failed to induce RANTES production by fibroblasts that did not exhibit Syk, and TNF-α induced RANTES production in an independent manner on the Syk-expression level. We also demonstrated that IL-1-signaling was associated with Syk and TRAF6, although TNF-α caused no association of TRAF2 of Syk. The artificial change of Syk expression affected JNK-phosphorylation and p38-phosphorylation by IL-1. The signal pathway of Syk-JNK or Syk-p38 connects with NF-κB activation in RANTES gene expression.

The IL-1 and TNF-α signals are transduced through TRAF6 and TRAF2, respectively (15). IL-1 induced the interaction of TRAF6 with IRAK, although IRAK did not associate with TRAF2 after the stimulation of IL-1 (14). The engagement of cytokine receptors with TRAF6 activates the antiapoptotic serine/threonine kinase (Akt/protein kinase B) through a signaling complex involving Src family kinases and TRAF6 (16). Tyrosine kinases could regulate the cytokine gene expression and IRAK activity (17). We revealed that IL-1 induced chemokine production from the nasal fibroblasts through a signaling complex involving Syk and TRAF6. IL-1 also induced the interaction of c-Src with TRAF6. Src homology (SH) 3 domains contain proline-rich motifs containing PXXP consensus sequence (24), and TRAF6 contains a SH3-interacting sequence (16). Therefore, Src family kinases have SH3 domains with the ability to interact with TRAF6. In human platelets, Syk associated transiently with c-Src, and Src family kinases participated in the signaling (25). In our system, Syk could take an important role with Src family kinases and TRAF6 in IL-1 signaling. However, we cannot exclude the possible interaction with unknown adaptor proteins to mediate this signaling.

IL-1 stimulated transcription factors activating protein-1 and NF-κB through activation of JNK and the IκB kinase (15). In response to IL-1, MAPK was activated in a concentration-dependent manner, and its activation was attenuated by tyrosine kinase inhibitor (13). In this report, we demonstrated that IL-1 induced the phosphorylation of JNK mainly and p38 partially in nasal fibroblast lines. RANTES mRNA expressions were induced through the activation of MAPKs (11–13) or through JNK and NF-κB kinase cascades in macrophages (26). The Syk-generated signal cooperates to enhance Rac-induced JNK activation in T lymphocytes (27). MAPKs activation was compromised in the macrophages of Syk−/− mice after Fc-γ receptor stimulation (28). The cytokine production was produced by an early tyrosine phosphorylation of Syk in murine resident peritoneal macrophages (29). Here, we found that Syk expression mainly affected JNK activation in response to IL-1 in nasal fibroblast lines. Transcriptional activation of the human RANTES promoter was dependent on specific activating protein-1, which was regulated by JNK (26). Syk has two SH2, instead of SH3, and the C-terminal SH2 domain of Syk was required for induction of JNK-activation (30).

Regulation of RANTES chemokine gene expression requires NF-κB through IκB (9). Recently, Das (31) reported that JNK mediated NF-κB activation after degradation of IκB. We demonstrated that Syk was related to JNK phosphorylation and degradation of IκB. Phosphoinositide 3-kinase and Akt were involved in NF-κB signaling (32). TRAF6 induced Akt activation through Src family kinases (16). Syk also is required for the activation of the phosphoinositide 3-kinase and Akt in B cells (33).

Toll-like receptor has been proven to mediate LPS-induced cellular signaling (34). TRAF6 mediated both IL-1- and LPS-induced signaling. However, TRAF2 did not mediate in the IL-1- and LPS-signaling while it was involved in TNF-signaling (35). We also found that pretreatment of antisense oligodeoxynucleotides to Syk inhibited RANTES production by fibroblasts stimulated with LPS (4).

The pathogenetic findings of nasal polyps shows an increase in infiltrating cells including eosinophils (36). Eosinophils and fibroblasts strongly interacted with each other in the pathogenesis of airway diseases, because fibroblasts are a rich source of chemokines, cytokines, and inflammatory mediators. Recently, Stenton et al. (37) reported that Syk antisense oligodeoxynucleotides delivered by aerosol to lungs in vivo depressed Syk expression and pulmonary inflammation. In conclusion, Syk proteins are endogenously expressed in human nasal fibroblasts. Syk antisense oligodeoxynucleotides depressed Syk expression and chemokine production in nasal fibroblasts that originated from nasal polyps. The regulation of Syk expression may prove useful as one of the strategies for the treatment of airway diseases such as asthma or nasal polyps.

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**References**