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Increased expression of L-plastin in nasal polyp of patients with nonsteroidal anti-inflammatory drug-exacerbated respiratory disease

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Abstract

Background: Most patients with nonsteroidal anti-inflammatory drug-exacerbated respiratory disease (NERD) suffer from recurrence of nasal polyps. However, little is known about the specific cellular and molecular mechanisms contributing to the pathogenesis of nasal polyp development in patients with NERD in particular, especially at baseline when cyclooxygenase 1 inhibitors are not present. The objectives of this study were to identify proteins involved in the pathogenesis of nasal polyps in patients with NERD.

Methods: We collected nasal polyp tissue from patients with NERD and from patients with aspirin-tolerant chronic rhinosinusitis with nasal polyps (CRSwNP). Protein profiles were analyzed by 2-dimensional electrophoresis and identified several proteins, including L-plastin, as highly expressed. We examined L-plastin and tissue factor (TF) expression by immunohistochemical and immunofluorescence analyses. To examine the role of L-plastin in eosinophils, we knocked down L-plastin expression in Eo1 cells by using siRNA transfection.

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AUTHOR CONTRIBUTIONS

TT, SF, and RPS designed the study. TT, DS, YI, KT, YK, and KY performed the experiments. YT performed MALDI-TOF MS analysis. MS analyzed data with the help of NN. TT, TN, and SH helped with sample collection and evaluation. All authors have read and approved the final form of the manuscript. TT and RPS wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Results: L-plastin protein levels in nasal polyp tissue were increased in patients with NERD relative to those in patients with aspirin tolerant CRSwNP. Immunofluorescence analysis revealed that L-plastin was dominantly expressed in eosinophils and L-plastin and TF were co-expressed in eosinophils in NERD nasal polyp tissue. Knockdown of L-plastin in Eo1-1 cells disrupted the cell surface distribution of TF by stimulation with granulocyte macrophage colony-stimulating factor.

Conclusion: Increased expression of L-plastin by eosinophils may contribute to abnormal fibrin deposition through TF translocation to the eosinophil cell surface in NERD nasal polyp tissue, which in turn may contribute to the pathogenesis of NERD.

GRAPHICAL ABSTRACT

Increased expression of L-plastin, the leukocyte-specific actin-bundling protein, co-expressed with TF in eosinophils in NERD nasal polyp tissue. L-plastin translocate TF to eosinophil cell surface, which in turn initiates extrinsic coagulation cascade by binding to FVIIa and induces subsequent excessive fibrin deposition in the nasal submucosa. L-plastin is also implicated in cytokine release or migration of eosinophils. TF, tissue factor; NERD, nonsteroidal anti-inflammatory drug-exacerbated respiratory disease; FVIIa, clotting factor VIIa.

1 | METHODS

1.1 | Patients and sample preparation

Patients with NERD and aspirin-tolerant controls with CRSwNP were recruited from the Department of Otorhinolaryngology Head & Neck Surgery of the University of Fukui and Department of Neck Surgery, Dokkyo Medical University. Nasal polyp tissues were obtained during routine functional endoscopic sinus surgery from subjects with NERD or from controls with CRSwNP. All patients met the criteria for CRS, as defined by the guidelines of the European position paper on rhinosinusitis and nasal polyps.¹ All subjects signed an informed consent, and the protocol and consent forms governing procedures for the study were approved by the institutional review board of the University of Fukui and Dokkyo Medical University. Further details are provided in the supporting information.

1.2 | Two-dimensional difference gel electrophoresis (2D-DIGE) and protein identification

Of 8 nasal polyp specimens of each NERD and aspirin-tolerant controls with CRSwNP were used. The subjects' characteristics are shown in Table S1. Labeling of samples for 2D-DIGE was performed according to the manufacturer's instructions (GE Healthcare, Fairfield, CT). Proteins were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Details are provided in this article's Methods section in the supporting information.

1.3 | Western blot analysis

Western blot analysis was performed using rabbit anti-human L-plastin polyclonal antibody (ab83496; Abcam, Cambridge, UK). Details are provided in this article's Methods section in the supporting information.

1.4 | Immunohistochemistry

Briefly, blocked sections were incubated with mouse anti-human L-plastin mAb (clone: LPL4A.1, IgG1, ab3290; Abcam) or mouse anti-human fibrin mAb (SEKISUI diagnostics, Stamford, CT) or rabbit anti-human tissue factor antibody (bs-4690R; Bioss Antibodies, Woburn, MA) at 4°C overnight. Details are provided in this article's Methods section in the supporting information. Subject characteristics are included in Table S3.

1.5 | Cell culture

The methods for culture of Eol-1 are detailed in this article's Methods section in the supporting information.

1.6 | L-plastin siRNA transfection

L-plastin and nonsilencing scrambled control siRNA were purchased from Thermo Fisher Scientific (Waltham, MA). Details are provided in this article's Methods section in the supporting information.

1.7 | Real-time PCR

Total RNA from cultured Eol-1 was extracted using NucleoSpin RNA II (MACHEREY-NAGEL, Bethlehem, PA) according to the manufacturer's instructions. Real-time RT-PCR was performed with a TaqMan method using an Applied Biosystems StepOnePlus™ System (Applied Biosystems, Foster City, CA). Details are provided in this article's Methods section in the supporting information.

1.8 | Statistical analysis

All data are reported as mean $\pm$ SEM unless otherwise noted. Differences between groups were analyzed with the Kruskal-Wallis ANOVA with Dunnett post hoc testing and Mann-Whitney *U* test. Correlations were assessed by using Spearman rank correlation. A *P* value of less than 0.05 was considered statistically significant.

2 | INTRODUCTION

Nonsteroidal anti-inflammatory drug-exacerbated respiratory disease (NERD) is characterized by a triad of hypersensitivity to aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), asthma, and chronic rhinosinusitis with nasal polyp (CRSwNP). The pathogenesis of NERD involves dysregulation of arachidonic acid metabolism, which results in excessive basal generation of the cysteinyl leukotrienes (CysLTs), leukotriene (LT) C₄, LTD₄, and LTE₄, which are further increased by exposure to aspirin/NSAIDs. These CysLTs are thought to contribute to several of the characteristic features of NERD.² Histologically, nasal polyps from patients with NERD are characterized by intense infiltration of eosinophils. Many patients with NERD suffer from recurrence of nasal polyps, and surgical intervention is frequently necessary to clear the nasal passages. Therefore, elucidation of the pathogenesis of nasal polyp formation has particular significance for improving the effect of NERD therapy.

Usually, nasal polyps arise from in and around the middle nasal meatus or paranasal sinuses. The major histopathological features of nasal polyps are intense edematous stroma and formation of pseudocysts filled with plasma proteins, mainly albumin.^{3,4} CysLTs are mediators of allergic inflammation, which causes vascular leakage that may contribute to intense edema in the nasal mucosa and cause development of nasal polyps.⁵ It has been reported that the levels of CysLTs in nasal polyps from patients with NERD were significantly higher than in polyps from patients with aspirin-tolerant asthma, and the levels of systemic CysLTs after endoscopic sinus surgery were significantly decreased in patients with NERD.⁶ Thus, it seems likely that nasal polyps are the main source of CysLTs in NERD patients. However, therapeutic responses to CysLT inhibitors are not uniform among patients with NERD, and antileukotriene drugs usually do not shrink or eliminate nasal polyps. Therefore, mediators other than CysLTs may also contribute to the pathogenesis of nasal polyps in NERD patients. We recently reported that the relative gene expression of tissue plasminogen activator (t-PA) was decreased in nasal polyps from patients with NERD relative to that in patients with CRSwNP.⁷ In a previous study, we hypothesized that reduction of tPA, a critical mediator in fibrinolysis, could in turn contribute to the excessive fibrin deposition and intense edema seen in nasal polyps.⁸ Therefore, further reductions in t-PA in NERD could potentially lead to greater enhancement of fibrin deposition and thus help explain the clinical observation that nasal polyps of patients with NERD grow more tenaciously and are more recalcitrant to medical and surgical treatment than are those of patients with aspirin-tolerant CRSwNP.^{9,10} Better understanding of the mechanisms underlying nasal polyp development might identify novel targets that could be used to prevent the serious consequences of NERD.

The objective of this study was to identify proteins involved in the pathogenesis of nasal polyps in NERD by employing a relatively new proteomic differential display method that uses fluorescence-based 2D-DIGE coupled with MALDI-TOF MS.¹¹ We found that L-plastin protein levels were upregulated in nasal polyps from patients with NERD compared to polyps from CRSwNP patients and examined the role of this protein in nasal polyp development.

3 | RESULTS

3.1 | L-plastin protein levels were upregulated in nasal polyps from patients with NERD

To identify proteins involved in the pathogenesis of nasal polyps in NERD, we examined the pattern of protein expression in NERD nasal polyp tissues by using a proteomics approach. Lysates of nasal polyp tissue from patients with NERD and patients with aspirin-tolerant controls with CRSwNP were prepared and labeled with fluorescent dyes. Image analysis of fluorescently labeled lysate loaded together on two-dimensional gels showed differences in the expression of some proteins between NERD and control CRSwNP nasal polyp tissues (Figure 1A). Protein spots that were increased by >2.0-fold were selected for MALDI-TOF identification. Sixty-one spots were differentially expressed (>2.0-fold increase; $P < 0.05$ on ANOVA) and analyzed by MALDI-TOF MS. Nine proteins were identified from the NCBI database search by using Mascot software (Matrix Science, Boston, MA, USA) (Table S2).

Among the 9 differential expressed proteins, we focused on L-plastin and further confirmed the finding by Western blotting in 6 nasal polyp samples (NERD, $n = 3$; CRSwNP, $n = 3$). The L-plastin protein was detected as a 67-kDa protein, and we found that the L-plastin band density was increased in the nasal polyps of the patients with NERD relative to that in the nasal polyps of patients with CRSwNP (1.045 ± 0.37 vs 0.560 ± 0.20) (Figure 1B). A beta-actin antibody was used to confirm equal sample loading.

3.2 | Immunohistochemical analysis of L-plastin in nasal polyp tissue

To further characterize the expression of L-plastin protein in patients with NERD, we performed immunohistochemical analysis of nasal polyp tissues from patients with NERD and aspirin-tolerant controls with CRSwNP to determine whether L-plastin expression could be detected. As shown in Figure 2A-C, we detected L-plastin staining mainly in submucosa inflammatory cells. We found that L-plastin+ inflammatory cell numbers were highly elevated in nasal polyps tissue from patients with NERD relative to those from control CRSwNP (Figure 2D). L-plastin is reported to be expressed primarily in leukocytes and regulates actin reorganization of inflammatory cells, such as eosinophils.^{12–14} We therefore focused on eosinophils and assessed whether the number of L-plastin+ inflammatory cells correlated with the number of eosinophils in nasal polyp tissues. We found that the number of L-plastin+ inflammatory cells in nasal polyp tissue was significantly and positively correlated with the number of eosinophils ($r = 0.765$, $P < 0.0001$; Figure 2E). We next performed dual-immunofluorescence analysis using anti-L-plastin and antibodies against a marker of eosinophils (ECP, eosinophil cationic protein). We found a high degree of colocalization of L-plastin within ECP+ eosinophils in the nasal polyps of the patients with NERD (Figure 3).

3.3 | Fibrin deposition in polyp tissue from patients with NERD

Although the intensity of the infiltration of nasal polyps with eosinophils is a defining feature of NERD,^{15,16} the precise role of eosinophils in the development of nasal polyps of patients with NERD has not been clarified. We have previously associated reduced t-PA with fibrin deposition and recently reported that expression of t-PA was more profoundly reduced in nasal polyps from patients with NERD relative to that in patients with CRSwNP.^{7,8} We

suggested that a reduction of t-PA, an important mediator in fibrinolysis, could contribute to excessive fibrin deposition and tissue remodeling, which in turn forms recalcitrant nasal polyps in NERD. To evaluate the fibrin deposition in nasal polyp tissue, we performed immunofluorescence analysis of surgical samples from patients with NERD and control CRSwNP (Figure 4A,B). Intense staining of fibrin was found in the submucosa of nasal polyp tissue from patients with NERD (Figure 4A). Fibrin staining intensity was graded by blinded observers, as described in this article's Methods section in the supporting information. The semiquantitative analysis showed significantly more intense fibrin staining in nasal polyp tissues from patients with NERD than from patients with CRSwNP (Figure 4C). We also found that the numbers of eosinophils were significantly and positively correlated with the intensity of fibrin staining in nasal polyp tissues ($r = 0.4637$, $P < 0.005$; Figure 4D). These results suggest that eosinophils might be involved in excessive fibrin deposition in NERD nasal polyp tissue.

3.4 | Detection of tissue factor (TF) in L-plastin+ eosinophils

Eosinophils were found to express TF, which initiates the extrinsic coagulation cascade and subsequent fibrin deposition.^{17,18} Binding of factor VIIa to TF on the surface of cells initiates the coagulation cascade by activating both factors IX and X, which in turn leads to thrombin generation and subsequent fibrin clot formation.¹⁹ The molecular mechanisms that regulate TF translocation on cell surfaces have not been completely elucidated. Activation of eosinophils has been reported to cause translocation of TF to the cell surface,¹⁷ and a recent study suggested that granulocyte-macrophage colony-stimulating factor (GM-CSF) induces activation of eosinophils via L-plastin phosphorylation and colocalization of L-plastin with F-actin at the leading edge of activated cells.^{13,14} We therefore hypothesized that L-plastin may regulate the distribution of TF in eosinophils. To examine whether eosinophils express TF in NERD nasal polyps, we performed immunohistochemical analysis of nasal polyp tissues from patients with NERD and CRSwNP. We detected TF staining of NERD nasal polyps mainly in mucosal epithelium and inflammatory cells (Figure 5A); staining in NERD was more intense than in control CRSwNP nasal polyps (Figure 5B). Furthermore, TF+ inflammatory cells appeared likely to be eosinophils (Figure 5A, right lower enlarged image). Dual-immunofluorescence analysis using anti-TF and antibodies against markers of eosinophils (ECP, eosinophil cationic protein) showed a high degree of colocalization of TF with eosinophils in NERD nasal polyps (Figure 5C). To further determine whether TF+ eosinophils were the L-plastin-expressing cells in NERD nasal polyps, we performed dual-immunofluorescence analysis using anti-TF and anti-L-plastin antibody. We found that TF colocalized with L-plastin (Figure 5D). These results suggested that TF and L-plastin were expressed simultaneously in eosinophils of NERD nasal polyps.

3.5. | L-plastin regulated translocation of TF to cell surfaces in EoL-1 cells

To examine the role of L-plastin in TF translocation in eosinophils, we knocked down L-plastin expression in EoL-1 cells and evaluated TF expression. Knockdown of L-plastin using siRNA was quantified by real-time polymerase chain reaction and ELISA (Figure S1A and B). EoL-1 cells were transfected with control or L-plastin siRNAs, subsequently stimulated with GM-CSF for 1 h, and TF distribution was observed by immunofluorescence analysis using an anti-TF antibody. Although GM-CSF stimulation induced translocation of TF to the

cell surfaces in both nontransfected and control siRNA transfected Eo1-1 cells, transfection with siRNA for L-plastin diminished the cell surface distribution of TF by stimulation with GM-CSF (Figure 6). These results suggested that L-plastin has a pivotal role in TF translocation to the surface of activated eosinophils.

3.6 | L-plastin knockdown attenuated transmigration of Eo1-1 across the endothelium

The participation of eosinophils in allergic inflammation requires their emigration into the tissue. It has been reported that L-plastin regulates the localization of F-actin at the leading edge and is implicated in polarization and migration in chemokine-stimulated eosinophils.¹² We thus tested whether silencing of L-plastin blocks eosinophil transmigration. We found that transfection with siRNA to knockdown L-plastin attenuated transmigration by stimulation with GM-CSF (Figure S1C). These data suggest that L-plastin might promote eosinophil emigration and interaction with the endothelial surface during GM-CSF-induced transendothelial migration.

4 | DISCUSSION

In this study, we used 2D-DIGE coupled with MALDI-TOF MS and identified 9 proteins that were upregulated at least twofold in nasal polyp tissues from patients with NERD compared to polyps from patients with aspirin-tolerant controls with CRSwNP (Figure 1A, Table S2). Among these proteins, we further investigated L-plastin, a leukocyte-specific actin-bundling protein that is a critical regulator of actin dynamics in cells of both the adaptive and innate immune systems.^{12,20} Peripheral blood and tissue eosinophilia is a prominent feature of NERD, and tissue eosinophils, especially in nasal polyp tissue, are profoundly activated.^{7,10} The transition of quiescent eosinophils to an activated state is referred to as priming and is accompanied by augmented adhesion, response to chemoattractants, transendothelial migration, etc.^{21,22} A recent study reported that phosphorylated L-plastin, through GM-CSF stimulation, has a critical role in priming eosinophils.¹⁴ The current study demonstrates for the first time that there is a profound elevation of L-plastin in NERD nasal polyp tissue eosinophils (Figures 2 and 3). Our earlier study also demonstrated that GM-CSF levels in nasal polyp tissues were modest but significantly elevated in NERD relative to control CRSwNP.⁷ Taken together, these data support a hypothesis that elevated GM-CSF may initiate priming of eosinophils through L-plastin phosphorylation, which might render eosinophils more susceptible to chemotaxis, degranulation, and cytokine production and thereby have an important role in the pathophysiology and development of NERD nasal polyps. Indeed, in the current study, we found that siRNA transfection for the knockdown of L-plastin expression in Eo1-1 cells significantly decreased the levels of cytokine release by stimulation with lipopolysaccharide or tumor necrosis factor- α (Figure S2).

Although CysLT overproduction is a hallmark of NERD, the mechanisms of their overproduction remain elusive. Several studies suggested that platelet adherent leukocytes, particularly eosinophils, enhance CysLT production via transcellular metabolism of arachidonic acid.^{23,24} Recently, it has been reported that the percentage of platelet adherent leukocyte is markedly increased in peripheral blood and nasal polyps, and correlated with

CysLT production in patients with NERD, and the mutual activation of platelets and eosinophils is required for this observation.²⁵ Activated platelets release GM-CSF, 4 separate subjects which activates eosinophils and subsequently leads to CysLT overproduction in platelets.²⁴ Therefore, increased levels of L-plastin might be involved in the imbalance of eicosanoid metabolism in patients with NERD.

Although nasal polyps from patients with NERD and CRSwNP are both defined by a predominantly type 2 inflammation environment, NERD nasal polyps are much more likely to have rapid relapse postoperatively.²⁶ Major histopathological features of nasal polyps are intense infiltration of inflammatory cells and prominent edematous stroma filled with plasma proteins, mainly albumin.³ We have previously reported that excessive fibrin deposition in nasal polyps caused by impairment of fibrinolysis and acceleration of the extrinsic coagulation cascade might play a critical role in forming prominent edema.^{8,27} Most recently, we demonstrated that Nattokinase, a serine protease possessing strong fibrinolytic activity, effectively shrunk nasal polyp tissue in vitro through fibrin degradation while papain, a well-known representative of the cysteine protease family, did not.²⁸ These results reinforced the view that profound fibrin deposition might be involved in the retention of plasma proteins and the formation of apparent tissue remodeling, intense edema in nasal polyp tissue. We also showed here that fibrin deposition was profoundly increased in nasal polyp tissue from patients with NERD relative to polyps from patients with CRSwNP (Figure 4A–C). We have previously found that nasal polyps from patients with CRSwNP have significantly decreased levels of t-PA, an important mediator of fibrinolysis, which could in turn contribute to the excessive fibrin deposition seen in this disease.⁸ Furthermore, our recent study found that the relative gene expression of t-PA was even lower in nasal polyp tissue from patients with NERD compared to polyps from patients with CRSwNP.⁷ Such profound reductions in t-PA in NERD nasal polyps could nearly completely eliminate the fibrinolytic mechanism and potentially lead to greatly enhanced fibrin deposition in nasal submucosa in polyp tissue. Increased fibrin mesh formation and reduced fibrinolytic mechanisms could help explain the clinical observation that NERD nasal polyps are more recalcitrant to medical and surgical interventions and more likely to relapse than are aspirin-tolerant CRSwNP.^{29–31}

Fibrin mesh, as the final product of the coagulation cascade, has a major role in blood clotting. In addition, activation of the coagulation cascade and extravascular fibrin mesh formation as a consequence of inflammation is well known and is thought to have a critical role in host defense.⁴ However, dysregulation of the coagulation cascade facilitates abnormal fibrin deposition and may have an etiologic role in many diseases, including rheumatoid arthritis, severe asthma, glomerulonephritis, delay-type hypersensitivity, and Crohn's disease.^{32–36} A recent study demonstrated that eosinophils are a major source of TF, the initiator of extrinsic coagulation cascade, in chronic urticaria and have a critical role in disease pathophysiology.³⁷ In the current study, we observed a significant positive correlation between the eosinophil numbers and fibrin deposition levels in nasal polyp tissues ($r = 0.4264$, $P < 0.005$; Figure 4D). We also found that TF+ inflammatory cells were increased, and most eosinophils expressed TF in nasal polyp tissue from patients with NERD (Figure 5A–C). Taken together, these data support a hypothesis that the upregulation

of TF in eosinophils has a critical role in the formation of excessive fibrin deposition, which might be involved in tissue remodeling in NERD nasal polyp tissue.

Although binding of TF to clotting factor VIIa on cell surfaces is necessary for initiation of extrinsic coagulation cascade, preformed TF is stored in intracellular compartments of eosinophils under resting condition.³³ Our immunohistochemical data demonstrated that TF staining was most prominently observed in the pericellular area of eosinophils (Figure 5A right lower panel). In the current study, we found that both L-plastin and TF were expressed in eosinophils of NERD nasal polyp tissue (Figure 5C,D), and silencing of L-plastin disrupted TF translocation to the cell surface of Eo1-1 by GM-CSF stimulation (Figure 6). It has also been reported that phosphorylation of L-plastin has a crucial role in the transport of the T-cell activation molecules CD69 and CD25 to the surface of T cells.³⁸ Activation of eosinophils causes translocation of TF to the cell surface,¹⁵ and a recent study suggested that GM-CSF induced activation of eosinophils via L-plastin phosphorylation and colocalization of L-plastin with F-actin at the leading edge of activated cells.^{12,13} Taken together, it is reasonable to speculate that increased levels of L-plastin might be involved in the translocation of TF to the eosinophil cell surface, which in turn initiates extrinsic coagulation cascade by binding to FVIIa and induces subsequent excessive fibrin deposition in the nasal submucosa of patients with NERD. However, the exact mechanisms by which L-plastin regulates TF distribution in eosinophils remain unclear. Several studies have demonstrated the interaction of the TF cytoplasmic domain with filamin A, a widely expressed actin-binding protein that regulates reorganization of the actin cytoskeleton, and TF colocalizes with filamin A in the leading edge of plasma membranes during cell migration.^{39–41} It is reasonable to speculate that phosphorylation of L-plastin increases its avidity for actin and cross-links actin filament into tight bundles, which increases the stability of actin-based structures that might be involved in TF translocation to cell surfaces through binding with filamin A, but this will require further experiments to test.

It has been reported that L-plastin regulates the localization of F-actin at the leading edge and is implicated in polarization and migration in chemokine-stimulated eosinophils.¹² In the current study, we found that silencing of L-plastin profoundly decreased the transendothelial passage of Eo1-1 (Figure S1C). A previous report also suggested that TF is one of the critical mediators of the initial eosinophil migration across the endothelium.⁴² On the basis of the above data, there is a possibility that the interaction of TF with L-plastin may have a critical role, possibly via binding with filamin A, in tissue eosinophilia, a typical clinical feature of NERD nasal polyps.

In summary, this study showed that L-plastin was profoundly increased in NERD nasal polyp tissue and that eosinophils were the major L-plastin-producing cells in nasal polyp tissue. We also found that the levels of TF, the initiator of the extrinsic coagulation cascade, were increased in eosinophils of NERD nasal polyp tissue and that L-plastin might be necessary to translocate TF to cell surfaces by GM-CSF stimulation, which would result in excessive fibrin deposition in the submucosa of NERD nasal polyp tissue. Because the interplay between L-plastin and TF also regulates eosinophil migration, targeting of L-plastin phosphorylation in eosinophils might be of therapeutic value for treating patients with NERD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

2D-DIGE	2-dimensional fluorescence-based difference gel electrophoresis
CRSwNP	chronic rhinosinusitis with nasal polyp
CysLTs	cysteinyl leukotrienes
GM-CSF	granulocyte-macrophage colony-stimulating factor
LT	leukotriene
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
MS	mass spectrometry
NERD	nonsteroidal antiinflammatory drug-exacerbated respiratory disease
NSAIDs	nonsteroidal anti-inflammatory drugs
TF	tissue factor
t-PA	tissue plasminogen activator

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FIGURE 1.

(A) Representative data from 2D-DIGE images of lysate nasal polyp tissue from a patient with NERD (left) and an aspirin-tolerant control with CRSwNP (right) using 12.5% SDS-polyacrylamide gel in the pH 4-7 range are shown. Protein spots that were increased >2.0-fold were selected for MALDI-TOF identification. Spot numbers (see Table S2) indicate significantly upregulated (>2.0-fold) proteins in NERD vs CRSwNP nasal polyps. (B) Western blot analysis of protein extracts from NERD and CRSwNP nasal polyps. Upregulation of L-plastin in NERD nasal polyp

FIGURE 2.

Immunohistochemistry of L-plastin using anti-human L-plastin antibody was performed. Representative immunostaining for L-plastin in nasal polyps from a patient with NERD (A) and aspirin-tolerant control with CRSwNP (B). Isotype control antibody staining in nasal polyp tissue from a patient with NERD (C) is shown. The numbers of L-plastin⁺ cells in nasal polyps from patients with NERD (n = 10) and aspirin-tolerant controls with CRSwNP (n = 28) were counted (D). (E) Correlation of the number of L-plastin⁺ cells with the number of eosinophils in nasal polyp tissue. The correlation in nasal polyp tissue was assessed by performing the Spearman rank correlation test. Magnification: $\times 400$. ** $P < 0.01$. HPF, high-power field

FIGURE 3.

Immunofluorescence of L-plastin in NERD nasal polyp tissue. Immunofluorescence assay using antiL-plastin (*red* fluorescence) and anti-ECP antibody (*green* fluorescence) was performed. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (*blue* fluorescence). The results are representative of 4 separate subjects

FIGURE 4.

Immunohistochemical analysis of fibrin in nasal polyp tissue using anti-human fibrin antibody was performed. Representative immunostaining for fibrin in nasal polyp tissue from a patient with NERD (A) and an aspirin-tolerant control with CRSwNP (B). (C) Semiquantitative analysis of fibrin in nasal polyp tissue from patients with NERD (n = 19) and patients with CRSwNP (n = 20) was performed. (D) Correlation between the degree of fibrin deposition and number of eosinophils in nasal polyp tissue. (*closed circles*, NERD; *open squares*, aspirin-tolerant control with CRSwNP). The correlation shown was assessed by performing the Spearman rank correlation test. Magnification: $\times 400$. * $P < 0.05$

FIGURE 5.

Immunohistochemical analysis of tissue factor in nasal polyp tissue by using anti-human tissue factor antibody was performed. Representative immunostaining for tissue factor in nasal polyp tissue from a patient with NERD (A) and a patient with CRSwNP (B). Immunofluorescence of tissue factor in NERD nasal polyp tissue. Immunofluorescence assays using anti-tissue factor (*green* fluorescence) and anti-ECP antibody (red fluorescence) were performed (C). The results are representative of 4 separate subjects. Immunofluorescence assays using anti-tissue factor (*green* fluorescence) and anti-L-plastin antibody (*red* fluorescence) were performed (D). The results are representative of 4 separate subjects

FIGURE 6.

L-plastin regulates the distribution of TF at the cell surface in GM-CSF-stimulated Eo1-1 cell. Eo1-1 cells were transfected with control or L-plastin siRNAs and subsequently stimulated with GM-CSF for 1 h. The cells were fixed and then measured for tissue factor immunofluorescence (*green*)