Nerve growth factor induces type III collagen production in chronic allergic airway inflammation

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Background: Excessive extracellular matrix deposition occurs as a result of repetitive injury-repair cycles and plays a central role in the pathogenesis of chronic inflammatory diseases, such as allergic asthma. The molecular mechanism leading to aberrant collagen deposition is not fully understood.
Objective: We sought to test the hypothesis that increased nerve growth factor (NGF) production contributes to collagen deposition in the airways during chronic allergic airway inflammation.
Methods: Antibody-blocking experiments were performed in an *in vivo* model for chronic allergic airway inflammation (allergic asthma), which is accompanied by matrix deposition in the subepithelial compartment of the airways, to study the profibrotic effect of NGF. The signaling pathways were delineated with *in vivo* and i*n vitro* studies in primary lung fibroblasts.
Results: Functional blocking of NGF in chronically affected mice markedly prevented subepithelial fibrosis. Transgenic overexpression of NGF in murine airways resulted in altered airway wall morphology with increased peribronchial collagen deposition and impaired lung physiology in the absence of inflammation. NGF exerted a direct effect on collagen expression in murine lung fibroblasts, which was mainly mediated through the activation of the receptor tropomyosin-related kinase A. NGF-induced collagen expression was dependent on downstream activation of p38 mitogen-activated protein kinase independent of the TGF-β1/mothers against decapentaplegic homolog (SMAD) pathway.
Conclusion: The results of this study demonstrate that NGF exerts profibrotic activities in the airways by inducing type III collagen production in fibroblasts independently of TGF-β1.
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Key words: *Allergic asthma, airway remodeling, type III collagen, nerve growth factor*

Excessive collagen deposition by fibroblasts is a prominent characteristic in a variety of chronic inflammatory diseases and is associated with loss of organ function.[1] It is considered the consequence of exaggerated wound repair after repetitive or chronic insult to the tissue.[1] During inflammation and tissue injury, growth factors are secreted by the injured epithelium and infiltrating immune cells and drive the subsequent wound-healing process.[2] The production and deposition of type III collagen by fibroblasts as part of the provisional matrix for epithelial cell migration and proliferation is immediately initiated on injury.[1] Allergic asthma represents a chronic inflammatory condition in which tissue remodeling and restructuring is of disease-limiting importance. In patients with chronic asthma, excessive matrix deposition manifests as subepithelial fibrosis in the airways and contributes to lung function decline in the progression of the disease.[3,4] TGF-β1 has been identified as a major profibrotic growth factor[5,6]; however, there is strong evidence that antagonizing TGF-β1 might not be sufficient to prevent disease pathology. Furthermore, profibrotic pathways acting independently of TGF-β1/mothers against decapentaplegic homolog (SMAD) signaling have been recently identified.[7,8]

Nerve growth factor (NGF), a prototypic member of the neurotrophin family, was initially described in promoting the survival and differentiation of neuronal cells.[9] In addition to this neuroprotective activity, there is increasing evidence supporting the role of NGF in repair mechanisms. NGF is actively secreted on injury and during inflammation from various epithelial and inflammatory cells. Its ability to accelerate wound-healing processes has been shown in several pathological situations[10] and rodent models of tissue repair.[11] In this context NGF promotes epithelial cell proliferation[12] and migration, as well as contraction of lung fibroblasts.[13] These events represent an important step in the repair process and link this growth factor to profibrotic mechanism. Therefore we hypothesized that as a consequence of repetitive induction of lung damage, NGF levels are strongly upregulated and participate in deregulated repair mechanisms that result in subepithelial fibrosis in chronic inflamed airways. To investigate this concept and explore the mechanisms of how NGF might promote collagen deposition in the airways, we used a well-established and well-characterized murine model of chronic allergic airway inflammation that demonstrates the pathological process of subepithelial fibrosis.[14]

METHODS

The methods used are described in detail in the Methods section in this article's Online Repository at www.jacionline.org.

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Abbreviations used

BALF: Bronchoalveolar lavage fluid
col3(α1): Type III collagen (α1) chain
ERK: Extracellular signal-regulated kinase
JNK: c-Jun terminal kinase
MAPK: Mitogen-activated protein kinase
MLF: Murine lung fibroblast
NF-κB: Nuclear factor κB
NGF: Nerve growth factor
OVA: Ovalbumin
siRNA: Small interfering RNA
SMAD: Mothers against decapentaplegic homolog
SURS: Systematic uniform random sampling
TrkA: Tropomyosin-related kinase A
WT: Wild-type

Animals

Female C57BL/6 (wild-type [WT]) mice obtained from Harlan Winkelmann (Borchen, Germany), $Ngfr^{tm1Jae}$ ($p75^{NTR\ exonIII-/-}$), mice on a C57BL/6 background purchased from Jackson Laboratory (Bar Harbor, Me), and transgenic mice encoding NGF (NGF-Tg) under the control of the lung-specific Clara cell secretory protein (CCSP) promoter[15,16] at 6 to 8 weeks of age were used for the experiments. NGF-Tg mice were mated with $p75^{NTR\ exonIII-/-}$ ($p75^{-/-}$) mice.[17] Mice were genotyped for the presence of the NGF construct or deletion of exon III of the p75 gene. All mice used for the experiments were maintained under pathogen-free conditions in a 12/12-hour light/dark cycle with food and water available *ad libitum*. Experimental procedures were approved by the local animal ethics committee.

Ovalbumin sensitization and challenge

Mice were sensitized by 3 intraperitoneal injections of 10 μg of ovalbumin (OVA; grade VI; Sigma-Aldrich, Hamburg, Germany) adsorbed to 1.5 mg of aluminum hydroxide (Pierce, Rockford, Ill) dissolved in 200 μL of PBS at days 1, 14, and 21. OVA aerosol challenges (1% OVA, grade V, Sigma) were performed for 1 or 4 weeks, with 2 consecutive challenges per week (see Fig E1 in this article's Online Repository at www.jacionline.org).[14] Experimental groups were either sham immunized and sham treated (PBS/PBS), OVA immunized and sham treated (OVA/PBS), or OVA immunized and either treated with a control IgG (OVA/IgG) or anti-NGF (OVA/anti-NGF). Antibody dose and route are described in detail in the Methods section of this article's Online Repository.

Measurement of lung mechanics

Lung mechanics were assessed by using the invasive flexiVent-System (SCIREQ, Montreal, Quebec, Canada). Measurements are described in detail in the Methods section of this article's Online Repository.

Assessment of leukocyte distribution in bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously described.[18]

Measurements of cytokines in bronchoalveolar lavage fluid

Measurement of cytokines is described in the Methods section in this article's Online Repository.

Lung histology and morphometric analysis

After bronchoalveolar lavage fluid (BALF) collection, the right lung was fixed in paraformaldehyde (6% wt/vol). Systematic uniform random sampling (SURS) was performed to obtain a representative collection of lung tissue samples.[19] After paraffin embedment, 3-μm sections were stained with hematoxylin and eosin. Collagen fibrils were detected by means of staining with Sirius Red (Sigma-Aldrich)/Fast Green (Rowley Biochemicals, Danvers, Mass). See the Methods section in this article's Online Repository for additional details on the methods used for collagen quantification.

Isolation of murine lung fibroblasts, RNA isolation, and analysis of mRNA expression

Murine lung fibroblasts of WT and $p75^{-/-}$ mice were isolated by enzymatic digestion and magnetic bead isolation. For signaling pathway analysis, cells were pretreated with specific inhibitors for ERK1/2 (20 μmol/L U0126; Calbiochem, Nottingham, United Kingdom) and p38 MAPK (20 μmol/L SB202190, Calbiochem) followed by stimulation with 100 ng/mL rhNGF (PeproTech, Hamburg, Germany). Col3(α1) mRNA expression was analyzed by means of real-time PCR. Cell isolation, RNA isolation, and mRNA expression analysis are described in detail in the Methods section in this article's Online Repository.

Construction of luciferase reporter plasmid

Genomic fibroblast DNA was used to generate a col3(α1) promoter reporter construct containing the flanking sequence 5′ to the transcriptional start ATG (+1) cloned into pGL4.10 (firefly luciferase) mammalian expression vector (Promega, Madison, Wis). Construction of luciferase reporter is described in detail in the Methods section in this article's Online Repository.

Transient transfection and reporter gene analysis

HEK293 cells were transfected with pcDNA-ratTrkA (WT and mutant K538R) using FuGene HD (Roche, Penzberg, Germany). After 24 hours, the pCol3(α1)-luc and pGL4.73 were co-transfected. Cell extracts were analyzed using the dual-luciferase reporter assay system (Promega). Transient transfection and reporter gene analysis are described in detail in the Methods section in this article's Online Repository.

RNA interference

RNAi knockdown experiments were performed using specific siRNAs for TrkA and SMAD4, both puchased from Qiagen. RNA interference is described in detail in the Methods section in this article's Online Repository.

Immunoprecipitation and immunoblot analysis

Cells were lysed in 50 mM Tris/HCl pH7.5, 150 mM NaCl, 1% NP-40, 1 × protease- and phosphatase- inhibitor mix (both Roche) and used for immunoprecipitation and immunoblot analyses. Immunoprecipitation and immunoblot analysis are described in the Methods section in this article's Online Repository.

Statistical analysis

Data analyses were performed with the Prism 4 Software package (GraphPad Software, Inc, San Diego, Calif). Bar graph data were expressed as means ± SEMs. The box and whisker plots represent the 10th, 25th, 50th (median), 75th, and 90th percentiles. Where appropriate, a 2-tailed Student *t* test was done. For multiple-group comparisons, 1-way ANOVA followed by the Tukey posttest was applied. *P* values of less than .05 were considered statistically significant.

RESULTS

Development of subepithelial fibrosis in experimental chronic asthma is NGF dependent

Well-established murine models of acute and chronic allergic airway inflammation were used to assess the involvement of NGF in the production and deposition of peribronchial collagen.[14] We observed that NGF levels were augmented during disease