

Connective tissue growth factor expression is regulated by histamine in lung fibroblasts: Potential role of histamine in airway remodeling

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Background: In the inflamed lung of allergic asthma, an aberrant injury-repair response is accompanied by structural changes in the airway, known as airway remodeling. TGF- β and its downstream mediator connective tissue growth factor (CTGF) are playing a key role in these processes, resulting in irreversible airway remodelling.

Objective: As histamine is a key mediator of allergic reactions, we investigated whether histamine is involved in airway remodeling.

Methods: The effect of histamine and TGF- β 1 on proliferation of lung fibroblast cells IMR-90 was studied by [³H]-thymidine proliferation assay. The regulation of CTGF by histamine and TGF- β 1 in lung fibroblasts was analyzed by RT-PCR, real-time PCR, Western blot analysis, and promoter analysis and characterized by specific histamine-receptor antagonists.

Results: Histamine and TGF- β 1 enhanced proliferation of lung fibroblast cells IMR-90. Both induced CTGF mRNA and protein expression with different time kinetics. Whereas TGF- β 1 induced maximal CTGF expression after 12 hours ($347\% \pm 23\%$), histamine-induced maximal CTGF expression was lower and delayed (maximum expression of $204\% \pm 11\%$ after 48 hours). Histamine and TGF- β 1 stimulated the CTGF promoter and the TGF- β -response element in the CTGF promoter. The histamine-induced CTGF expression was mediated through the histamine receptor (HR1) and could be completely abolished by TNF- α .

Conclusions: These findings demonstrate that histamine plays a potential role in the induction of airway remodeling mediated by the induction of lung fibroblasts proliferation and CTGF expression.

Clinical implications: This mechanism could be important for prophylactic strategies aiming at airway remodeling and could be a new indication for antihistamine treatment. (*J Allergy Clin Immunol* 2007;119:1398-407.)

Key words: Asthma, CTGF, Smad signaling, TGF- β 1, vascular remodeling

Although asthma is considered to be an entirely reversible disorder, patients with asthma experience an accelerated rate of deterioration of respiratory function.^{1,2} This deterioration is clinically characterized by irreversible or only partially reversible airway obstruction and persistent airway hyperresponsiveness. Recent attention has been directed to the structural changes in the airways, which are collectively referred to as airway remodeling. These changes include basement membrane thickening, alterations in the composition of the extracellular matrix (ECM), epithelial desquamation, inflammatory cell infiltration, an increase in the number of blood vessels, and smooth-muscle cell and lung fibroblast proliferation.³

Although lung fibroblasts are important in generating a structural framework for the lung, they also seem to be active participants in the inflammatory and remodeling process via proliferation and the production of specific mediators.⁴ Furthermore, fibroblasts are responsible for the production of ECM components and may be precursors for various cell types including myofibroblasts.⁴ The production of inflammatory and fibrotic mediators by lung fibroblasts is especially important in relation to their strategic location within connective tissue as cellular communication bridging interstitium and vasculature in the lung.

TGF- β is one critical mediator in the pathogenesis of asthma, which is involved in both the regulation of airway inflammation and airway remodeling.⁵ Increased TGF- β 1 levels were detected in asthmatic bronchoalveolar lavage fluids.⁶ TGF- β 1 acts as suppressive cytokine that prevents lymphocyte activation and mediates peripheral tolerance toward allergens.⁷ In addition, TGF- β 1 assists during wound repair inducing ECM deposition and thus contributes critically to lung fibrosis.⁸ Also TGF- β 2, expressed mainly by eosinophils, is expressed in severe asthma and is associated with increased profibrotic responses.⁹ In the chronically inflamed lung, prolonged

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Abbreviations used

CTGF: Connective tissue growth factor
ECM: Extracellular matrix
HR: Histamine receptor

activation and expression of TGF- β act as a continuous stimulus to repair inflammation-induced tissue destruction leading to an irreversible remodeling of the airways.¹⁰ Downstream of TGF- β 1, connective tissue growth factor (CTGF) modulates the cellular response to TGF- β 1 and contributes to wound healing and fibrotic response.¹¹ CTGF stimulates fibroblasts proliferation and ECM synthesis, and overexpression of CTGF is linked to many fibrotic diseases.¹²

Mast cells are known to play a central role in the immediate phase reaction of allergic diseases through the IgE-mediated release of a variety of chemical mediators like histamine. Higher histamine levels were found in plasma and bronchoalveolar lavage of subjects with allergic asthma compared with control subjects without asthma.¹³ The interaction of histamine with the HR1 mediates a variety of classic pathophysiologic effects, including vascular permeability, smooth-muscle contraction, vasodilation and flushing, mucus secretion, and pruritus.¹⁴ In addition to these well-characterized effects in the acute allergic responses, it has been shown that histamine affects chronic inflammation and regulates several essential events in the immune response.^{15,16}

Little is known of the modulation of airway remodeling either by therapeutic intervention, such as the use of long-term corticosteroids,¹⁷ or by endogenous factors, like histamine. In this study, we examined whether histamine interacts with lung fibroblasts with respect to fibroblast growth, activation, cytokine and ECM protein release, and subsequent extracellular fibrosis.

METHODS

Reagents

Recombinant human TGF- β 1, IFN- γ , and TNF- α were obtained from R&D Systems (Abingdon, United Kingdom [UK]). Histamine, triprolidine, and ranitidine were purchased from Sigma (St Louis, Mo).

Tissue cultures

Human lung fibroblasts (IMR-90, ATCC-CCL-186; American Type Culture Collection ATCC, Rockville, Md) were grown in the Dulbecco modified Eagle medium supplemented with 3.7-g/L sodium bicarbonate, 10% (vol/vol) fetal bovine serum, 100 U/mL penicillin, 10 μ g/mL streptomycin, 0.1 mmol/L Na-pyruvate, and 0.1 mmol/L nonessential amino acids. After confluence, serum content of the medium was reduced to 0.2% fetal bovine serum for 48 hours; these cells were quiescent. Cells were used in experiments between passage number 3 and 20. No significant differences were observed between early and late passage lung fibroblasts for CTGF mRNA and promoter induction and cell proliferation in response to TGF- β 1 and histamine. Cell numbers were determined by triplicate cell counts with an electronic particle counter (Coulter Counter ZM;

Coulter Corp., Hialeah, Fla). For inhibition, experiments cells were preincubated for 30 minutes with specific histamine receptor (HR) antagonists before histamine was added.

Viability detection

IMR-90 cell viability after exposure to TGF- β 1 (10 ng/mL), histamine (1 mmol/L), triprolidine (10^{-3} mol/L), ranitidine (10^{-3} mol/L), TNF- α (10 ng/mL), or IFN- γ (500 U/mL) was evaluated after 1-5 days using flow cytometry-based quantification of ethidium bromide (1 μ mol/L; Sigma Aldrich) uptake as previously described.¹⁸

Lung fibroblasts proliferation assay

IMR-90 lung fibroblasts were incubated in 96-well plates (Costar, Corning, NY) in the absence or presence of different concentrations of TGF- β 1 and/or histamine. Triplicates of IMR-90 cells (3×10^3 /well) were incubated for 5 days. Cells were pulsed for 16 hours with 1 μ Ci [^{3} H]-thymidine (Hartmann, Braunschweig, Germany) and harvested on glass fiber filters using an automated multisample harvester (LKB, Pharmacia-Wallac, Turku, Finland). Filters were transferred in sample bags with liquid scintillation fluid and analyzed using a β -scintillation counter (Pharmacia-Wallac). Results are expressed as counts per minute.

Flow cytometric analysis

IMR-90 fibroblasts were prepared for flow-cytometric analysis by washing a single-cell suspension of cells in PBS with 0.1% azide and 1% BSA. Cells were incubated with a fluorescein isothiocyanate (FITC)-labeled mouse antihuman CD40 mAb (MCA1590F; Serotec, Oxford, UK) for 30 minutes on ice. FITC-labeled mouse IgG2a was used as negative control purchased from Serotec (MCA929F). The cells were washed, resuspended in PBS with 0.1% azide and 0.1% BSA, and analyzed on a Coulter Elite flow cytometer (Coulter Corp.). Trypsinization, required for the fibroblasts, did not affect the expression of CD40 (data not shown). Viable cells were gated on the basis of forward light scatter, and data were analyzed with the Cytologo software program (Coulter Corp.). Results are expressed as a percentage of positive cells or in median fluorescence intensity (MFI) values after subtraction of the MFI obtained with the control mAb. All experiments were repeated a minimum of 3 times.

Quantitative real-time and direct RT-PCR analysis of mRNA

Total RNA was isolated from IMR-90 lung fibroblasts using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was eluted in 40 μ L water and subjected to reverse transcription. Overall, 5 μ g total RNA was reverse transcribed by addition of 500 μ g/mL oligo (dT)₁₂ primers (Roche, Basel, Switzerland), RNase Inhibitor (Roche) (10 U/ μ L), dNTP (5 mmol/L each dNTP; Qiagen), and Omniscript transcriptase (Qiagen) (0.2 U/ μ L) for 1 hour at 37°C. The cDNA was denatured at 90°C for 5 minutes and used for PCR amplification. PCR reactions were performed with Taq-polymerase (Qiagen). RT-PCR was performed using the primers listed in Table E1 in this article's Online Repository at www.jacionline.org. PCR products were loaded next to a standard (1kbpplus; Invitrogen Life Technologies, Paisley, UK) and analyzed on 1% agarose gels. Image analysis was performed using a fluorescence imager analyzer FLA 3000 (Fuji, Dielsdorf, Switzerland) and quantified using the AIDA software (Raytest, Urdorf, Switzerland).

For quantitative real-time RT-PCR analysis, 2 mL of undiluted cDNA were used per reaction; a standard curve for CTGF and β -actin

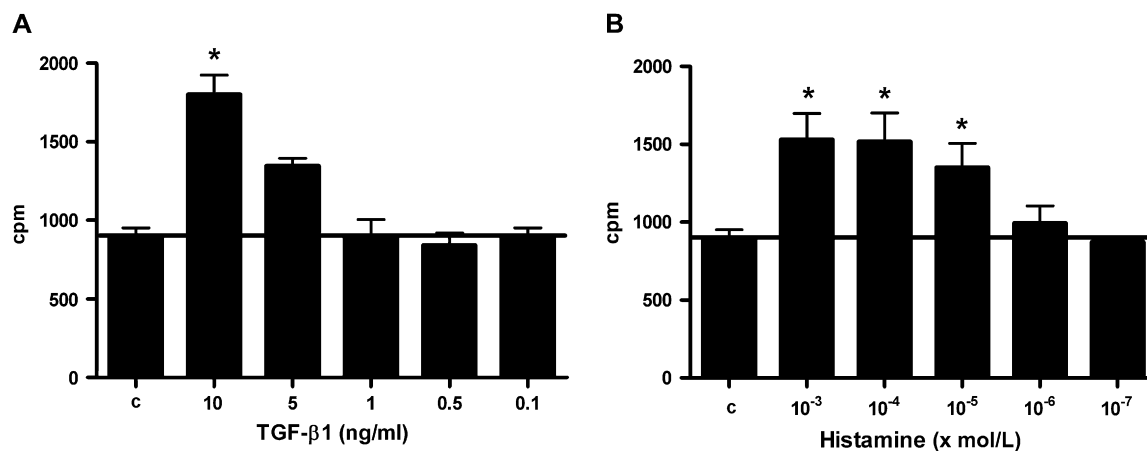


FIG 1. Effect of histamine and TGF- β 1 on the proliferation of lung fibroblasts. Proliferation was assessed 5 days after the addition of TGF- β 1 (**A**) or histamine (**B**) at the indicated concentration. Values are means $\pm$ SEMs for 3 replicate cultures. * $P < .05$ compared with control.

was generated from serial dilutions of positively expressing cDNA. The probe and primer sets were “predesigned assay on demand” probes (Applied Biosystems, Foster City, Calif). These predesigned primers were tested and standardized for reproducible expression analysis. Primer and cDNA were added to Taqman universal master mix (Applied Biosystems) containing all the reagents for PCR. Relative quantification of the PCR products was carried out using the ABI prism 7000 (Applied Biosystems) and using the comparative threshold cycle (C_T) method. The “fold-induction” was calculated as ratio to values of cells, which were not incubated with TGF- β 1 or histamine.

Western blot analysis

Analysis of CTGF expression and Smad2 phosphorylation in IMR-90 cells was performed by Western blot. Cell lysates (25 μ g) were boiled and loaded next to a molecular weight marker (Benchmark; Invitrogen Life Technologies) on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) under reducing conditions. The proteins were electroblotted onto a nitrocellulose membrane (Amersham Life Science, Dübendorf, Switzerland) under reducing conditions. Ponceau S staining of blots after transfer revealed equivalent loading of total protein. Subsequently, membranes were incubated with a goat anti-CTGF (sc-14939; Santa Cruz Biotechnology, Santa Cruz, Calif) or with a rabbit anti-Smad2-P antibody (Dr C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), and a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences), diluted at 1:10,000, was used as secondary antibody. Then specific reactive proteins were detected using enhanced chemiluminescence (Perbio, Bonn, Germany). Monoclonal anti- β -actin antibody (Santa Cruz Biotechnology) was used as primary antibody to control the amount of loaded protein after stripping the blot. The blot was visualized with a LAS 1000 camera (Fuji, Tokyo, Japan). Accumulated signals were analyzed using AIDA software (Raytest).

Transfection and promoter assays

Cloning of the CTGF promoter (pCT-sb)-, pAP-1-luc-, pTGF- β -RE-luc- and the (CAGA)₁₂-Luciferase plasmid were described before.^{19,20} pCT-sb (2 μ g), pAP-1-luc (2 μ g), pTGF- β -RE-luc (2 μ g), (CAGA)₁₂-luc (2 μ g), and *Renilla* luciferase control reporter vector (phRL-TK; 5 ng) were transfected into IMR-90, seeded in 6-well plates, with LipofectaminPlus (Invitrogen). Transfection medium (Optimem-medium, Invitrogen) was changed to 0.2% fetal bovine serum after 2 hours. Twenty-four hours after transfection, cells

were treated with indicated molecules. Then 16 hours later, luciferase activity was measured by the dual luciferase assay system (Promega Biotech Inc., Madison, Wis) according to the manufacturer’s instruction using a Berthold MiniLumat LB 9506 luminometer (Bad Wildbach, Germany). Firefly luciferase activity was normalized by the activity of *Renilla* luciferase under the control of thymidine kinase promoter of phRL-TK. Results are given as relative light units. All values were obtained from experiments carried out in triplicates and repeated at least 3 times. The error bars indicated standard error of the mean (SEM).

TGF- β 1 determination

Acid-activated TGF- β 1 was measured using the solid-phase sandwich ELISA with a sensitivity of less than 10 pg/mL (R&D Systems). Acid activation of supernatant was performed by the addition of 1 N HCl to reach pH 2.0 for 1 hour at 4°C. The supernatant was then neutralized using 1 N NaOH and used for ELISA. Control medium was treated in the same way.

Statistical analysis

All results shown are representative of 3 separate experiments. Results are presented as means $\pm$ standard errors of the mean (SEMs). The data were tested for significance using the Student-Newman-Keuls test. P values less than .05 were considered to be significant.

RESULTS

Histamine and TGF- β 1-enhanced lung fibroblast proliferation

To investigate whether histamine and TGF- β 1 influence the proliferative behavior of structural cells, the effect of histamine and TGF- β 1 on the *in vitro* proliferation of human lung fibroblasts was analyzed (Fig 1). Both TGF- β 1- and histamine-enhanced lung fibroblast proliferation in a dose-dependent manner. At the highest concentration tested, TGF- β 1 (10 ng/mL) enhanced proliferation of lung fibroblasts to $200\% \pm 12\%$ and histamine (1 mmol/L) to $170\% \pm 19\%$. Compared with medium, TGF- β 1 and histamine had no influence on the percentage of viable

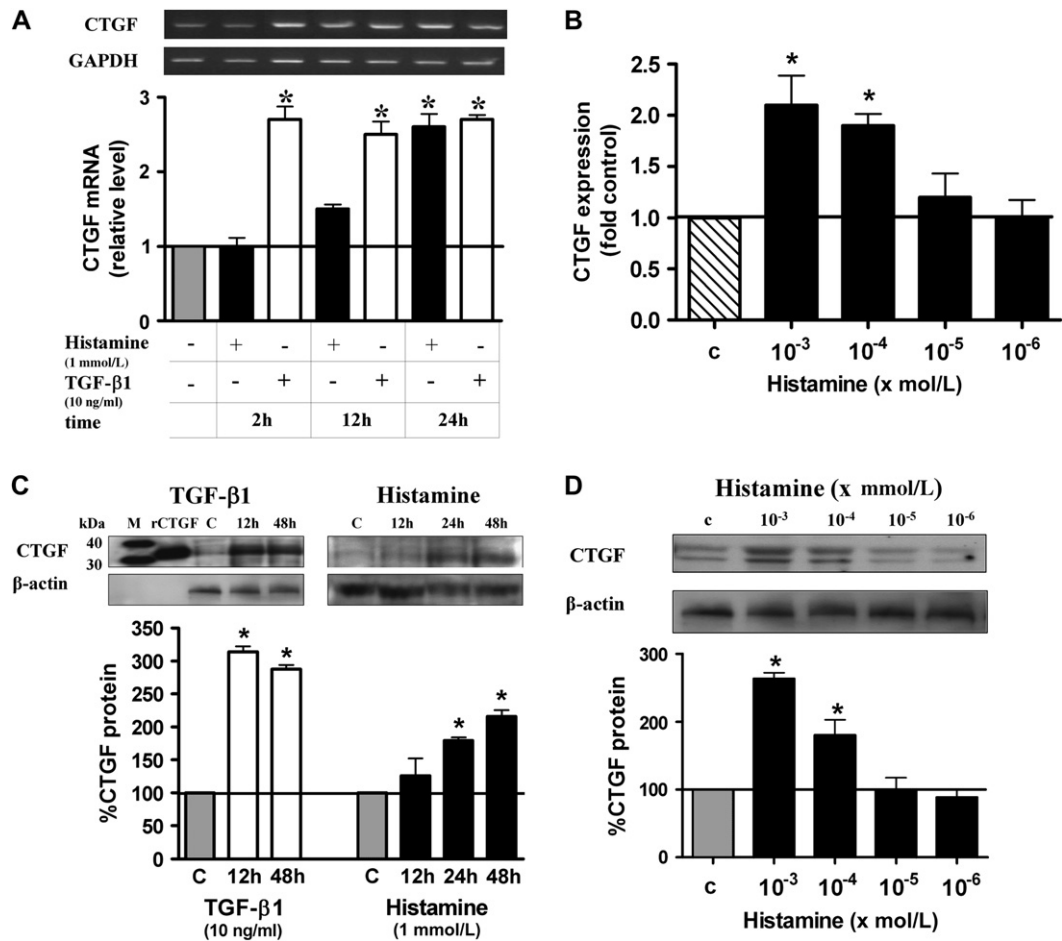


FIG 2. Histamine induces CTGF expression in lung fibroblasts on mRNA and protein level. **A** and **B**, Fibroblasts were stimulated with TGF-β1 and with histamine for the times indicated (**A**) or with different concentrations of histamine for 24 hours (**B**). Controls (*c*) represent untreated fibroblasts. CTGF mRNA expression was determined by semiquantitative competitive RT-PCR analysis (**A**) or by real-time RT-PCR (**B**). The *upper panel* shows electrophoretic visualization of amplicons, and the *lower panel* shows GAPDH-normalized quantification of the PCR products (**A**). Data are means ± SEMs of 3 independent experiments. **P* < .05 compared with control. **C** and **D**, Fibroblasts were stimulated with TGF-β1 or histamine. **C** shows the time dependency of TGF-β1 (*left side*) and histamine (*right side*) induction of CTGF protein expression. **D** shows the concentration dependency of histamine-induced CTGF protein expression (incubation time: 48 hours). Control (*c*) represents untreated fibroblasts. The *upper panel* shows the Western blots, and the *lower panel* shows the densitometric analysis of the relative increase of CTGF protein in cell lysates samples compared with control and normalized to β-actin. Three independent experiments were performed; representative blots are shown. **P* < .05 compared with control.

lung fibroblasts (medium/TGF-β1 [10 ng/mL]/histamine [1 mmol/L]: 1 day: 95%/97%/93%; 3 days: 88%/94%/86%; 5 days: 80%/83%/81%).

Histamine-stimulated CTGF mRNA and protein expression in lung fibroblasts

CTGF functions as a downstream mediator of TGF-β1 action on connective tissue cells and CTGF expression is induced by TGF-β1.¹¹ To evaluate the effect of histamine on the CTGF mRNA expression in lung fibroblasts, RT-PCRs were performed. Both histamine and TGF-β1 induced the mRNA expression of CTGF (Fig 2, A). Compared with TGF-β1 with a maximal increase of

2.7-fold after 2 hours, the effect of histamine on the CTGF mRNA expression was delayed, which increased after 12 hours (1.6-fold increase) and reached a maximum after 24 hours with a 2.6-fold increase. To verify our semiquantitative competitive RT-PCR results, we used real time RT-PCR. Real-time RT-PCR also showed that histamine stimulated a dose-dependent increase in the CTGF mRNA expression in lung fibroblasts after 24 hours (Fig 2, B).

The observed difference in the time course between TGF-β1 and histamine on CTGF transcription level could also be found at level of protein expression. A 1.8-fold increase in CTGF level was observed after a 24-hour

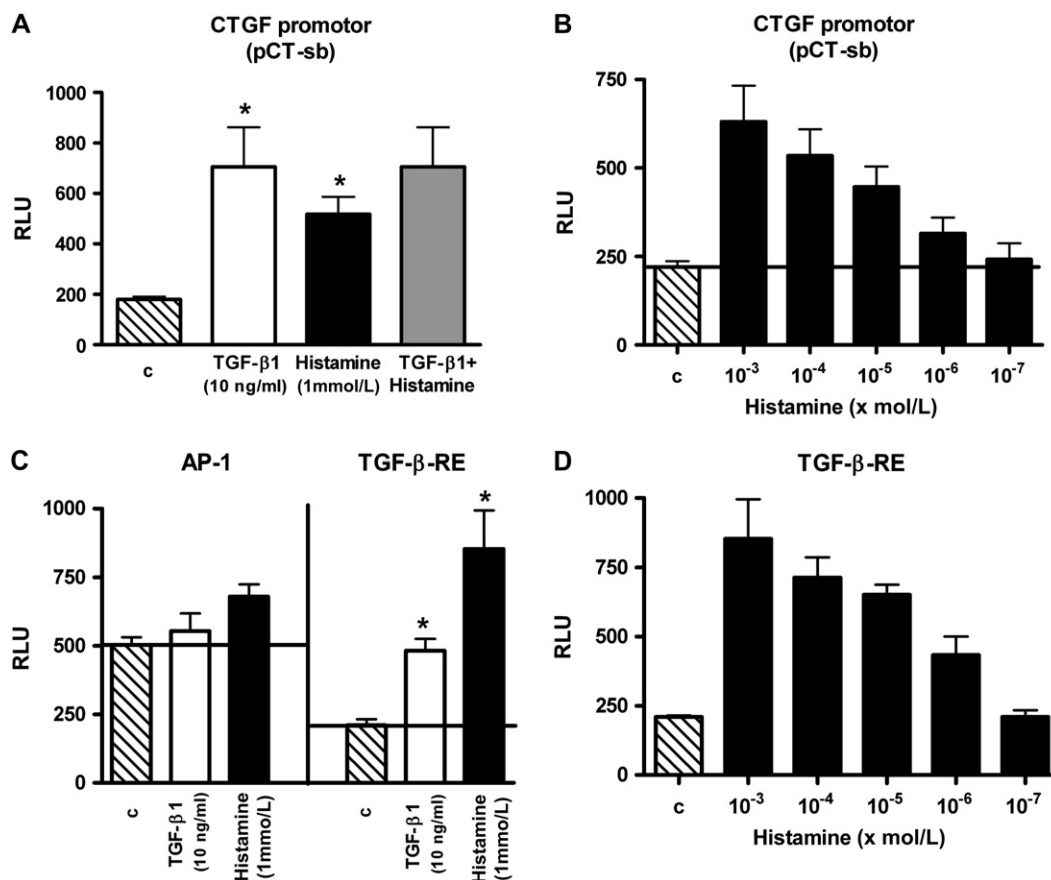


FIG 3. Histamine stimulates the CTGF promoter and the TGF- β -response element in the CTGF promoter. **A** and **B**, The CTGF promoter pCT-sb was transiently transfected into lung fibroblasts. Cells were then treated with TGF- β 1 or/and histamine for 24 hours (**A**). **B** shows concentration dependency of histamine-induced pCT-sb-luc reporter activity. **C** and **D**, Luminometric analysis of AP-1- or TGF- β -RE-luc reporter transfected lung fibroblasts, incubated with TGF- β 1 or histamine for 24 hours (**C**). **D** shows concentration dependency of histamine-induced TGF- β -RE-luc reporter activity. * $P < .05$ compared with control.

treatment with histamine, reaching maximal levels after 48 hours with a 2.1 increase (Fig 2, C). In the TGF- β 1-treated cells, the maximum of CTGF expression was already reached after 12 hours with a 3.1-fold increase (Fig 2, C). Histamine stimulated a dose-dependent increase in the protein expression of CTGF in lung fibroblasts after 48 hours (Fig 2, D).

Histamine stimulated the CTGF promoter and the TGF- β -response element in the CTGF promoter

To study the mechanism of CTGF induction by histamine, lung fibroblasts were transfected with the promoter of the CTGF gene (pCT-sb) and treated with histamine and as positive control with TGF- β 1. Luciferase activity was enhanced up to 2.8-fold in lung fibroblasts treated with histamine and up to 3.8-fold in TGF- β 1-treated cells (Fig 3, A), which shows that histamine acts via increasing the CTGF promoter activity. No additive effect could be found between TGF- β 1 and histamine on pCT-sb promoter activity (Fig 3, A). The histamine-induced stimulation of the pCT-sb promoter was dose dependent (Fig 3,

B). Histamine and TGF- β 1 had no effect on the promoter activity of the control pGL3 basic vector (data not shown).

Several regulatory elements have been identified in the 5'-untranslated region of CTGF, including a binding site for activating protein 1 (AP-1) and a unique TGF- β -response element (TGF- β -RE).²¹⁻²³ Transient transfection of these constructs in lung fibroblasts showed that only the TGF- β -RE, but not the AP-1 element, mediated the effect of histamine and TGF- β 1 on CTGF promoter activity. The histamine effect on the activity of the TGF- β -RE element was even stronger with a 4.0-fold increase than the effect of TGF- β 1 with a 2.3-fold increase (Fig 3, C). The histamine-induced stimulation of the TGF- β -RE element was dose-dependent (Fig 3, D).

Histamine did not affect TGF- β 1 synthesis and activation of Smad signaling

To exclude the possibility that histamine-mediated CTGF expression in lung fibroblasts is indirectly mediated by histamine-induced TGF- β 1 syntheses, the effect of histamine on TGF- β 1 mRNA expression and TGF- β 1 protein release was analyzed. No alteration in the histamine and

TGF- β 1-treated cells was observed for TGF- β 1 mRNA expression (see Fig E1, A in this article's Online Repository at www.jacionline.org). Furthermore, histamine did not increase the TGF- β 1 concentration in the supernatant of histamine-treated lung fibroblasts (see Fig E1, B in this article's Online Repository at www.jacionline.org).

TGF- β 1-mediated induction of CTGF gene expression in fibroblasts involves also a functional Smad binding site in the CTGF promoter.²⁴ To investigate the possible effect of histamine on Smad-signaling in lung fibroblasts, the TGF- β 1-sensitive (CAGA)₁₂-luciferase construct was used. The (CAGA)₁₂-luciferase construct was originally found in the plasminogen activator inhibitor-1 and is known to be activated after binding of Smad3/4 complexes and after TGF- β 1 binding to the TGF- β receptor.²⁰ The highest used concentration of 1-mmol/L histamine did not enhance the reporter gene activity of the (CAGA)₁₂-luciferase construct, whereas the positive control TGF- β 1 induced a 168-fold increase (see Fig E1, C in this article's Online Repository at www.jacionline.org). Along with Smad3, Smad2 is phosphorylated by TGF- β 1 and Smad2 phosphorylation can be tested as alternative evidence of Smad signaling. To determine whether Smad2 phosphorylation could be activated by histamine, lung fibroblasts were cocultured with TGF- β 1 or histamine and Smad2 phosphorylation was detected by immunoblotting with anti-phospho-Smad2-specific antibodies. TGF- β 1, but not histamine, could induce Smad2 phosphorylation in lung fibroblasts (see Fig E1, D in this article's Online Repository at www.jacionline.org).

CTGF expression by histamine was mediated by HR1

The expression of the HR subtypes 1-3 was tested in IMR-90 lung fibroblasts. The mRNA for HR1 and HR2 subtypes, but not for the HR3 subtype, was detected (Fig 4, A). The mRNA of the HR1 subtype was 8-fold higher expressed than the mRNA of the HR2 subtype.

To identify the histamine subtype responsible for histamine-induced CTGF expression, an HR1-specific and an HR2-specific antagonist were used. We compared the effect of histamine on CTGF protein level (Fig 4, B) and CTGF promoter activity (Fig 4, C and D) with and without the addition of the HR1 antagonist triprolidine or the HR2 antagonist ranitidine. The histamine effect on CTGF protein expression and CTGF promoter activity could be antagonized by the HR1 antagonist triprolidine, whereas the HR2 antagonist ranitidine had no effect.

Histamine-induced CTGF expression was reversed by TNF- α

TNF- α has been previously described to suppress the TGF- β 1-induced expression of CTGF and to down-regulate gene expression of CTGF.²⁵ We analyzed whether TNF- α also influences histamine-mediated CTGF expression. We compared the effect of histamine on CTGF protein level (Fig 5, A) and CTGF promoter activity (Fig 5, B) with and without the addition of TNF- α . Preincubation

with TNF- α for 2 hours before addition of histamine completely abolished the ability of histamine to induce CTGF protein induction (Fig 5, A) and pCT-sb and TGF- β -RE promoter activity (Fig 5, B).

Regulation of CD40, tenascin, IL-6, and IL-8 in lung fibroblasts by histamine and TGF- β 1

The CD40-CD40L interaction is a key element in controlling inflammation and fibrosis.^{26,27} The influence of histamine and TGF- β 1 on the expression of CD40 on lung fibroblasts was therefore tested. Lung fibroblasts were exposed to IFN- γ and/or histamine and TGF- β 1 and CD40 expression was analyzed by flow cytometry (see Fig E2, A in this article's Online Repository at www.jacionline.org). A total of $4.0\% \pm 1.2\%$ of untreated lung fibroblasts was stained positive for CD40. Culturing the lung fibroblast for 72 hours with IFN- γ resulted in a 13-fold increase in mean fluorescence intensity and resulted in $53.0\% \pm 2.9\%$ of lung fibroblasts expressing surface CD40. Histamine and TGF- β 1 did not induce CD40 expression and did not affect IFN- γ -induced CD40 expression. To evaluate the effect of histamine and TGF- β 1 on the mRNA expression of the ECM glycoprotein tenascin and the proinflammatory cytokines IL-6 and IL-8 in lung fibroblasts, RT-PCRs were performed using specific primer for these genes (see Table E1 in this article's Online Repository at www.jacionline.org). Incubation of lung fibroblasts with TGF- β 1 induced the expression of tenascin with a maximal increase of 3.5-fold after 2 hours (see Fig E2, B in this article's Online Repository at www.jacionline.org), the expression of IL-6 with a maximal increase of 53-fold after 24 hours (see Fig E2, C in this article's Online Repository at www.jacionline.org), and the expression of IL-8 with a maximal increase of 28-fold after 12 hours (see Fig E2, D in this article's Online Repository at www.jacionline.org), whereas histamine had no effect on the mRNA expression of these genes.

DISCUSSION

The current study demonstrated that histamine plays a role in allergic immune response as a contributor to airway remodeling. This finding is documented by the enhancement of proliferation and the induction of CTGF expression in lung fibroblasts by histamine. The CTGF expression was mediated by the HR1 and could be completely abolished by TNF- α . Promoter analysis showed that histamine activated the CTGF promoter and the TGF- β -RE element in the CTGF promoter. Compared with TGF- β 1, histamine-induced CTGF protein expression was lower and delayed.

The induction of CTGF expression in lung fibroblasts by histamine provides a new link between histamine and airway remodeling (see Fig E3 in this article's Online Repository at www.jacionline.org). The exact role of CTGF in airway remodeling in asthma has not been elucidated, but it has the potential to be a key factor in the development and maintenance of the structural changes

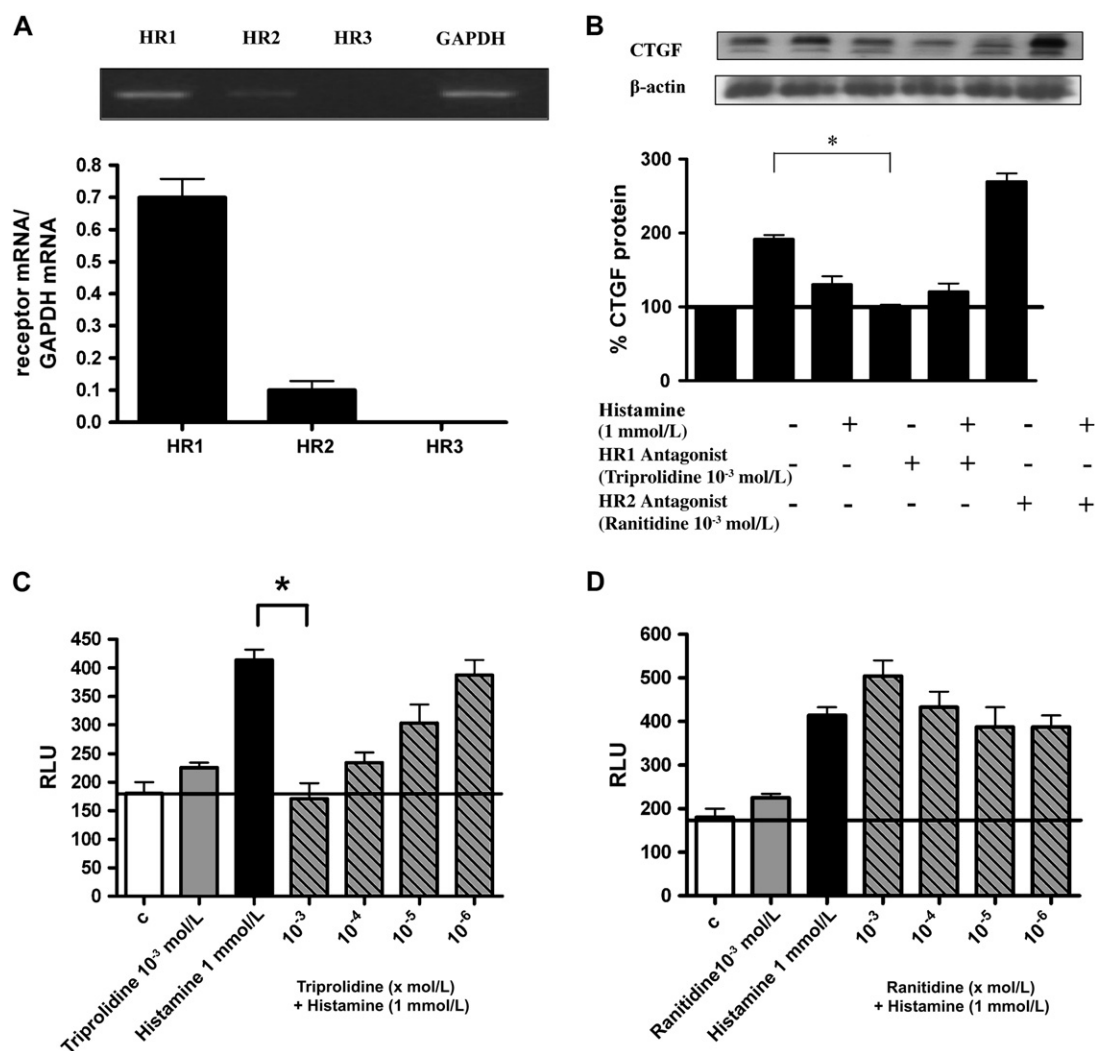


FIG 4. CTGF expression by histamine is mediated by HR1. **A**, The mRNA expression of the HR1-3 in lung fibroblasts was determined by RT-PCR analysis. The *upper panel* shows electrophoretic visualization of amplicons, and the *lower panel* shows GAPDH-normalized quantification of the PCR products. The *bars* represent the means of 3 independent experiments, including SEM. **B**, The effect of HR1 and HR2 antagonists on histamine-induced CTGF protein expression was studied by Western blotting. Lung fibroblasts were incubated with histamine, the HR1 antagonist tripolidine, the HR2 antagonist ranitidine, and histamine together with tripolidine or ranitidine for 48 hours. CTGF protein levels were determined by Western blotting. The *upper panel* shows the Western blots, and the *lower panel* shows densitometric analysis of the relative increase of CTGF protein in cell lysates samples compared with control and normalized to β -actin. Data are representative of 3 different experiments. * $P < .05$ compared with histamine alone. **C** and **D**, Luciferase reporter analysis of CTGF promoter pCT-sb-luc transfected in lung fibroblasts and incubated with histamine for 24 hours in the presence of different concentrations of the HR1 antagonist tripolidine (**C**) or the HR2 antagonist ranitidine (**D**). * $P < .05$ compared with histamine alone.

associated with severe persistent asthma.¹² TGF- β 1 specifically induces mRNA and protein for CTGF in airway smooth muscle, and the CTGF response was greater in airway smooth muscle cultured from patients with asthma compared with patients without asthma.²⁸ In addition to the influence of CTGF on connective tissue cells, CTGF also affects endothelial cell function, angiogenesis, and vascular remodeling.²⁹ Airway microvascular changes are also established as remodeling features typical of asthma.³⁰ Furthermore, CTGF may anchor vascular endothelial growth factor to the ECM, and this deposition is

decreased by matrix metalloproteinase-2 and prostaglandin E₂. This relationship could have the potential to contribute to the changes that constitute airway remodelling.³¹

Compared with TGF- β 1, the effect of histamine on CTGF expression in lung fibroblasts was less potent. This result is in line with the observation that other known growth factors, such as platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, or VEGF are less-potent stimuli for CTGF expression compared with TGF- β 1.^{12,32}

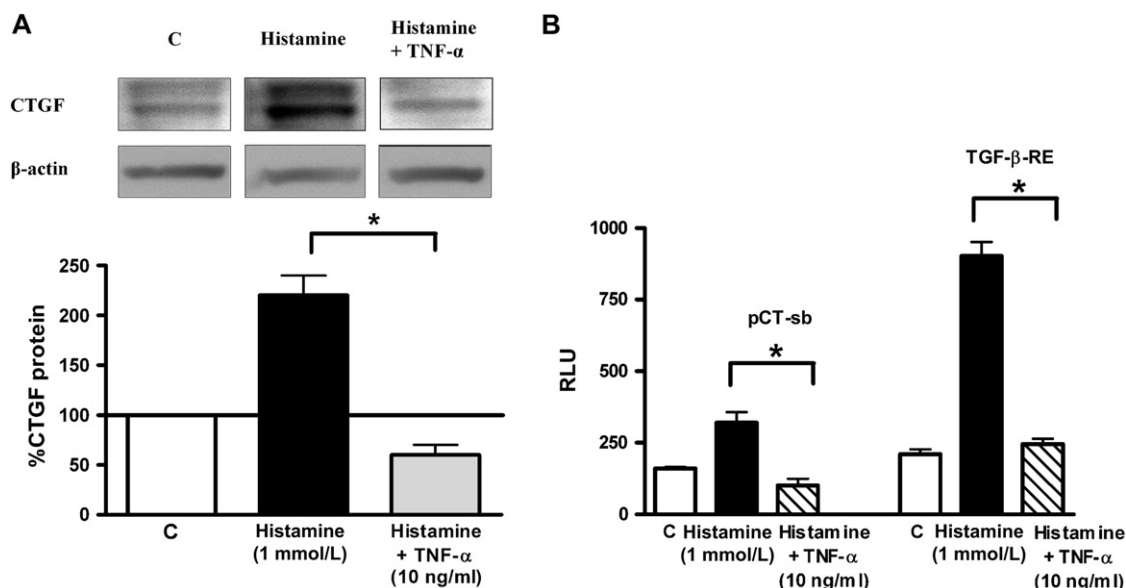


FIG 5. TNF- α inhibits the induction of CTGF by histamine. **A**, Lung fibroblasts incubated with histamine or TNF- α and histamine together for 48 hours. Control (c) represents untreated fibroblasts. The upper panel shows the Western blots, and the lower panel shows densitometric analysis of the relative increase of CTGF protein in cell lysates samples compared with control and normalized to β -actin. All values were obtained from experiments performed in triplicate, and the error bars indicate SEMs. * $P < .05$ compared with histamine alone. **B**, Luminometric analysis of pCT-sb-luc or TGF- β -RE-luc reporter transfected lung fibroblasts, incubated with histamine or histamine and TNF- α . * $P < .05$ compared with histamine alone.

To evaluate the signaling pathway leading to the induction of CTGF by histamine in lung fibroblasts, it could be shown that histamine induces the CTGF promoter and the TGF- β -RE in the CTGF promoter. This TGF- β regulatory element is located between positions 162 and 128 of the CTGF promoter sequence.²¹ It could be excluded beforehand that the effect of histamine on CTGF expression is indirectly mediated by the release of TGF- β 1. In addition, histamine did not activate Smad signaling in lung fibroblasts.

As described for TGF- β -induced CTGF expression,²⁵ histamine-induced CTGF expression was downregulated by TNF- α , a major proinflammatory cytokine. The role of TNF- α in the pathogenesis of severe asthma is not yet clear; however, 2 recently published studies reported an upregulation of TNF- α in patients with refractory, severe asthma compared with patients with mild-to-moderate asthma and control subjects.^{33,34} In the moment, one can only speculate that the detected increased TNF- α level could act as a natural protection factor against airway remodeling or a possible defect in the activation of the TNF- α axis could lead to airway remodeling in asthma patients.

Besides IL-4, nitric oxide, prostaglandin E₂, forskolin, and cyclic adenosine monophosphate can downregulate TGF- β 1-induced CTGF expression.¹²

Although histamine can activate the adenylate cyclase-cAMP/PKA pathway, mediated by H2R binding,³⁵ we demonstrate an upregulation of CTGF by histamine. Using specific H1R and H2R antagonists, it could be shown that the histamine-induced CTGF expression was mediated through the HR1.³⁵ HR1 stimulation results in

activation of phospholipase C and subsequently increases the second messengers inositol-1,4,5-triphosphate (IP₃) and intracellular calcium. HR1 signaling is involved mainly in allergic reactions related to smooth muscle contraction and vascular permeability.³⁶

Besides CTGF induction in lung fibroblasts, histamine contributes to airway remodeling via enhancement of lung fibroblasts proliferation. Histamine and TGF- β 1 have previously been shown to enhance the proliferation of lung fibroblasts.^{10,37-39} In addition, histamine increased proliferation, migration, and collagen production in conjunctival fibroblasts derived from vernal keratoconjunctivitis patients and healthy donors.⁴⁰

As the CD40/CD40-ligand system is a key regulator of fibroblasts activation, allowing interactions to the immune system, we investigated the impact of histamine and TGF- β 1 on CD40 expression. In the inflammatory microenvironment, fibroblasts are stimulated to upregulate CD40.²⁶ CD40 activation induces fibroblasts to proliferate, produce cytokines, and repair the injured tissue by releasing ECM components. IFN- γ , a cytokine found at high levels at sites of inflammation, upregulates the expression of CD40 on human fibroblasts *in vitro*.²⁶ As described before,²⁶ we found an upregulation of CD40 by IFN- γ on lung fibroblasts, but neither histamine nor TGF- β 1 induced an upregulation of CD40 and both of them did not influence IFN- γ -mediated upregulation of CD40. In addition, lung fibroblasts may potentially contribute to airway remodeling via the release of ECM proteins such as tenascin. The subepithelial basement membrane tenascin layer in the lung was significantly thicker in atopic

compared with nonatopic subjects with asthma.⁴¹ As described before, tenascin was induced *in vitro* by TGF- β 1 in lung fibroblasts.⁴² However, we did not find an induction of tenascin by histamine. Besides CD40, fibroblasts trigger the immune system and thus airway remodeling by IL-6 and IL-8 expression, which however was not regulated by histamine, whereas TGF- β 1 was confirmed as potent and induces these genes.⁴³ IL-6 and IL-8 are pleiotropic cytokines, often produced in prodigious amounts in inflammatory responses, and have been shown to be autocrine growth factors for pulmonary fibroblasts.⁴⁴

The used concentrations of TGF- β 1 and histamine seem to be adequate to mimic a possible biological effect of TGF- β 1 and histamine *in vitro*, especially to imitate the effect of both mediators in lung interstitium during acute allergic immune reactions. The TGF- β 1 and histamine concentrations in blood, plasma, and bronchoalveolar lavage fluid may be not representative concentrations of TGF- β 1 and histamine finding in lung interstitium, where the concentrations can be much higher, especially during allergic immune reaction, because of the close vicinity of mast cells and lung fibroblasts.^{45,46} The lung fibroblast cell line IMR-90 is adequate to mimic the processes during airway remodeling in the lung, because IMR-90 cells also have characteristics of myofibroblasts,⁴⁷ which is as an essential cell-type during airway remodeling.³

Until now, HR1 antagonists are very useful in the treatment of allergic rhinoconjunctivitis.³⁶ Although current evidence does not support the use of HR1 antagonists in persistent asthma, some investigators have shown a significant decrease in asthma symptoms and improvement in pulmonary function after HR1 antagonist treatment.^{15,36} This observation could be, in part, explained by an inhibition of histamine-induced CTGF expression by HR1 antagonists. The observation that histamine promotes the accumulation of CTGF could open a new window of therapeutic opportunity against airway remodeling in asthma based on histamine HR1 antagonist administration. Thus, HR1 antihistamines not only diminish the acute allergic reaction but may also abrogate fibrotic airway remodeling processes in chronic inflamed lung, which requires additional clinical evaluation.

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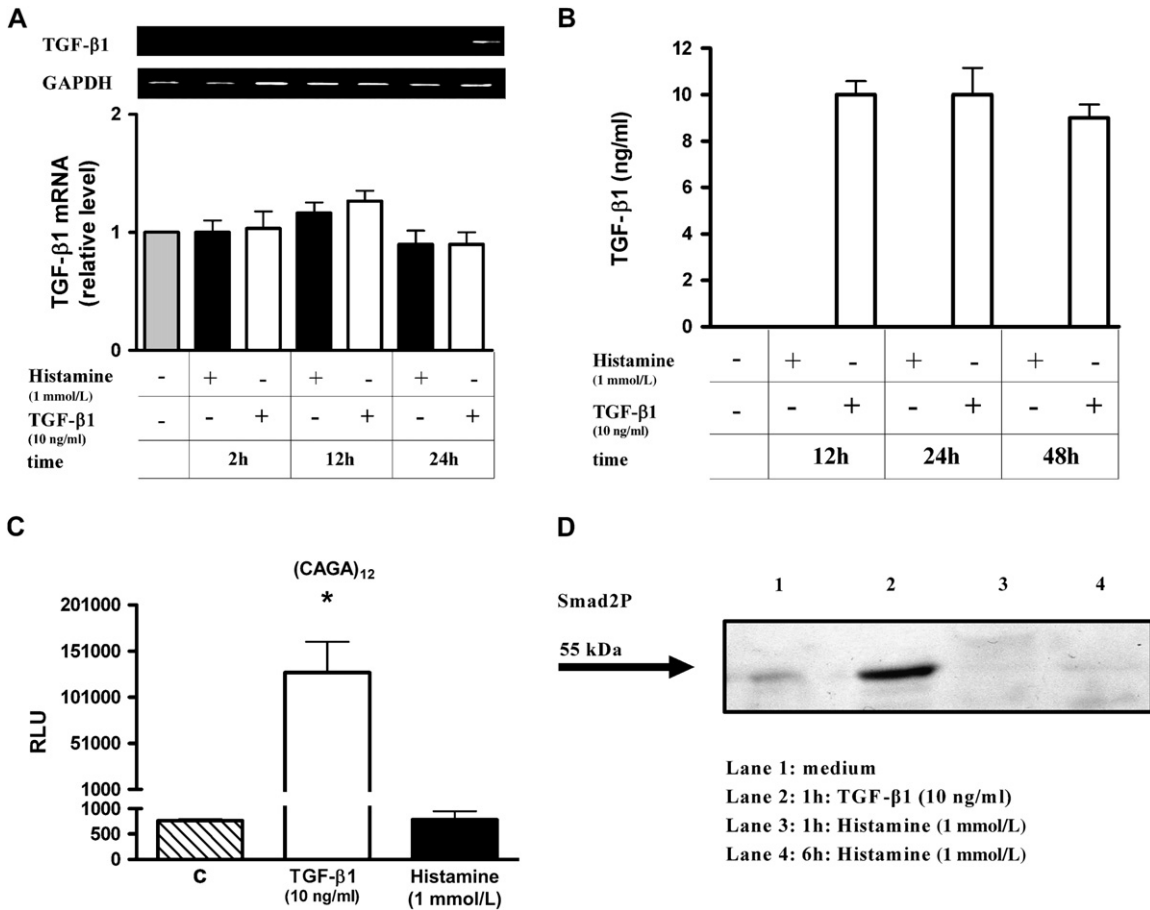


FIG E1. Histamine does not affect TGF-β1 synthesis and does not activate Smad signaling. **A**, mRNA expression of TGF-β1 in lung fibroblasts was determined by RT-PCR analysis. Cells were incubated with histamine or TGF-β1 for the indicated time. The *upper panel* shows electrophoretic visualization of amplicons, and the *lower panel* shows GAPDH-normalized quantification of the PCR products. Data are representative of 3 independent experiments. * $P < .05$ compared with control. **B**, Detection of TGF-β1 by ELISA. The concentration of TGF-β1 in the cell supernatant of TGF-β1 or histamine-treated lung fibroblasts were measured by ELISA at different time points. **C**, Luminometric analysis of (CAGA)₁₂-luc reporter transfected lung fibroblasts, incubated with TGF-β1 or histamine. * $P < .05$ compared with control. **D**, Phosphorylation of endogenous Smad2 protein. Lung fibroblasts were stimulated with TGF-β1 for 1 hour and with histamine for 1 hour and 6 hours. Smad2 phosphorylation was detected by immunoblotting with anti-phospho-Smad2 antibodies. A representative of 3 independent experiments is shown.

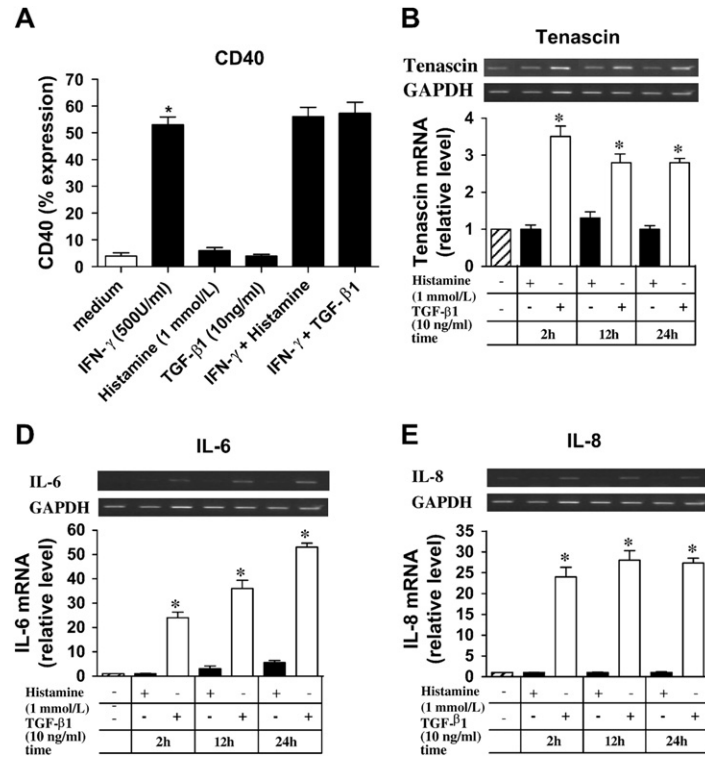


FIG E2. Regulation of CD40, tenascin, IL-6, and IL-8 in lung fibroblasts by histamine and TGF- β 1. **A**, Lung fibroblasts were or were not exposed to INF- γ and/or histamine and/or TGF- β 1. After 72 hours, CD40 expression was analyzed by fluorescence-activated cell sorting. Values are means $\pm$ standard errors of the mean for 3 replicate cultures. * P < .05 compared with control. **B-D**, mRNA expression of tenascin, IL-6, and IL-8 in lung fibroblasts was determined by RT-PCR analysis. Cells were incubated with histamine or TGF- β 1 for indicated time. The upper panel shows electrophoretic visualization of amplicons, and the lower panel shows GAPDH-normalized quantification of the PCR products. Data are representative of 3 independent experiments. * P < .05 compared with control.

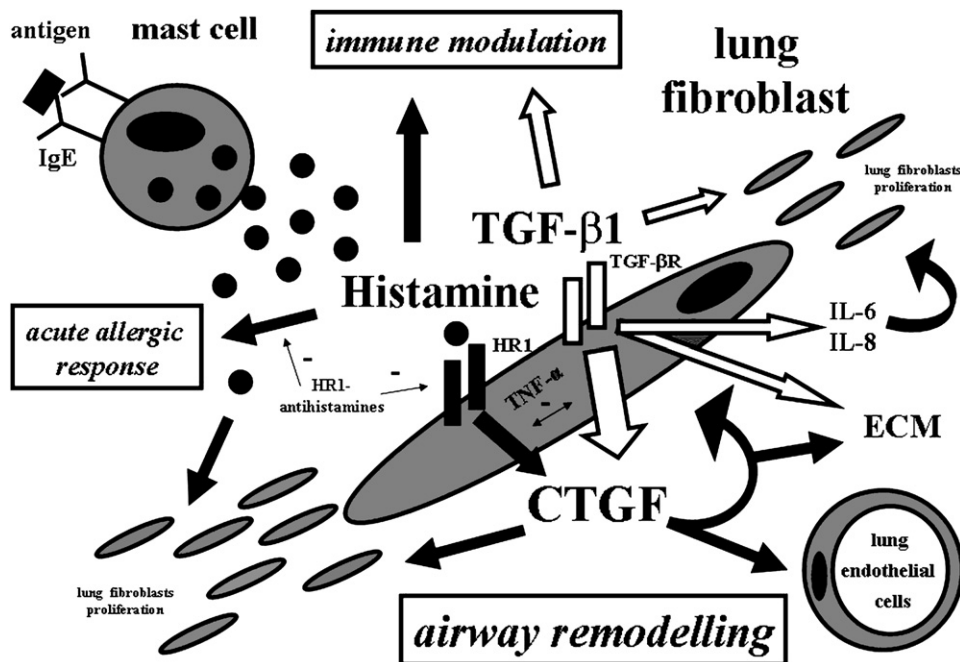


FIG E3. Schematic presentation of the role of histamine, TGF- β 1, and CTGF in airway remodeling. TGF- β 1 is one major cytokine in the pathogenesis of asthma, which is involved in both the regulation of airway inflammation and airway remodeling. TGF- β 1-enhanced proliferation of lung fibroblasts and expression of the profibrotic mediator CTGF, the proinflammatory cytokines IL-6 and IL-8 and extracellular matrix molecules. This study identified histamine as a new factor in airway remodeling, which increases the pathophysiologic understanding of histamine in asthma beyond its known role in the acute allergic reaction and immune modulation. Histamine is secreted by mast cells upon activation. Histamine stimulates proliferation of lung fibroblasts and induces the expression of CTGF, which is a key mediator of airway and vascular remodeling. The histamine-induced CTGF expression was mediated through the HR1, which could be completely abolished by HR1 specific blockers and TNF- α . Our study suggests that HR1 antihistamines not only diminish the acute allergic reaction but may also abrogate fibrotic airway remodeling processes in chronic inflamed lung.

TABLE E1. Primers used in this study

Primer	Sequence	Denaturation	Annealing	Extension	Cycle no.
		Temperature/ time	Temperature/ time	Temperature/ time	
CTGF forward	5' ACGGCGAGGTCATGAAGAAGAACA 3'	94°C/45 s	61°C/45 s	72°C/60 s	40
CTGF reverse	5' TGGGGCTACAGGCAGGTCAGTG 3'				
Tenascin forward	5' TGA ACA AAA TCA CAG CCC AG 3'	94°C/60 s	60°C/60 s	72°C/60 s	23
Tenascin reverse	5' CAG TGG AAC CAG TTA ACG CC 3'				
IL-6 forward	5' GCC CAG CTA TGA ACT CCT TCT C 3'	94°C/60 s	60°C/60 s	72°C/60 s	30
IL-6 reverse	5' GAG TTG TCA TGT CCT GCA GCC 3'				
IL-8 forward	5' ATG ACT TCC AAG CTG GCC GTG GCT 3'	94°C/60 s	60°C/60 s	72°C/60 s	30
IL-8 reverse	5' TCT CAG CCC TCT TCA AAA ACT TCT C 3'				
TGF- β 1 forward	5' GAC ATC AAC GGG TTC ACT ACC GG 3'	94°C/60 s	55°C/60 s	72°C/60 s	28
TGF- β 1 forward	5' GAG GCA GAA GTT GGC ATG GTA GC 3'				
HR1 forward	5' TAAGCTGAGGCCAGAGAACC 3'	94°C/60 s	60°C/30 s	72°C/60 s	35
HR1 reverse	5' TACTGTCTTGAATGCGAGCG 3'				
HR2 forward	5' GCACAGCCTCCATTCTTAACC 3'	94°C/60 s	60°C/30 s	72°C/60 s	35
HR2 reverse	5' CCTCATTGATGGCATCATCC 3'				
HR3 forward	5' TCCTCTGCCTTCAACATCG 3'	94°C/60 s	60°C/30 s	72°C/60 s	35
HR3 reverse	5' ATCATCAGCAGCGTGTATGG 3'				
GAPDH forward	5' CTT CGC TCT CTG CTC CTC CT 3'	94°C/60 s	60°C/60 s	72°C/60 s	27
GAPDH reverse	5' GCT GAT GAT CTT GAG GCT GTT G 3'				