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Histamine inhibits differentiation of skin fibroblasts into myofibroblasts

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ABSTRACT

Histamine and TGF-β, major mediators secreted by mast cells, are involved in skin inflammation and play critical roles in the pathogenesis of systemic sclerosis. However, the roles of signaling mechanisms in the development of skin fibrosis remain largely unclear. Here we show that histamine suppressed the expression of α smooth muscle actin (αSMA), a marker of myofibroblasts, induced by TGF-β1 in skin fibroblasts. Histamine H1-receptor (H1R), but not H2-receptor (H2R) or H4-receptor (H4R), was expressed on skin fibroblasts at both mRNA and protein levels. Interestingly, an H1R antagonist, but not H2R or H4R antagonists, antagonized the histamine-mediated suppression of αSMA expression by TGF-β1. Correspondingly, phosphorylated Smad2 was detected after treatment with TGF-β1, whereas the addition of histamine inhibited this phosphorylation. Taken together, histamine-H1R decreased TGF-β1-mediated Smad2 phosphorylation and inhibited differentiation of skin fibroblasts into myofibroblasts.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease of autoimmune origin characterized by proliferative vasculopathy and excessive deposition of extracellular matrix (ECM) [1,2]. In particular, abnormal deposition and remodeling of ECM constituents, including collagen type I, II, VII, and elastin, result in skin fibrosis; the extent of which is related to mortality rate in SSc [3,4]. Skin fibrosis is probably triggered by persistent activation of inflammatory immune cells, including dendritic cells, T cells, macrophages [5], and mast cells [6], which ultimately results in malfunction of skin tissue, and indeed, mast cells have been identified in fibrous tissues [7].

Mast cells are involved in several autoimmune pathologies through intracellular interactions. In a series of studies, we demonstrated the involvement of the cytokine-induced PDK/Akt1 pathway in mast cells survival [8], that mast cell-derived tryptase inhibits apoptosis of human rheumatoid synovial fibroblasts, and also the involvement of mast cell-derived transforming growth factor beta (TGF-β) in regulatory T cell generation during graft-versus-host disease [9,10].

Considered collectively, these results suggest the critical involvement of mast cells in the physiological and pathological processes of fibrosis, not only by direct interaction with fibroblasts, but also by the secretion of a series of crucial fibrogenic mediators, including histamine and TGF-β [11,12], which result in accelerated formation of dermal sclerosis that occurs particularly at an early phase of the sclerotic process [13]. However, there is little or no information on the interaction between TGF-β1 and histamine in the development and progression of skin fibrosis.

The TGF-β-family signaling molecules are multifunctional cytokines that contribute to tissue fibrosis [14], and comprise three members with similar structures and functions: TGF-β1, -β2, and -β3. TGF-β1/Smad signaling is the most crucial pathway involved in collagen formation and fibroblast differentiation into myofibroblasts. TGF-β1 binds to TGF-β receptor I (TGFβRI), resulting in recruitment to TGFβRII, which directly phosphorylates Smad2 and Smad3. The activated Smad2 and Smad3 then associate with Smad4

Abbreviations: αSMA, α smooth muscle actin; H1R, Histamine H1 receptor; H1RA, H1R antagonist; H2R, Histamine H2 receptor; H2RA, H2R antagonist; H4R, Histamine H4 receptor; H4RA, H4R antagonist; SSc, Systemic sclerosis.

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and translocate to the nucleus, where the complex regulates transcriptional responses of various target genes, including *αSMA* [15], the expression of which is a marker of skin fibroblast differentiation into myofibroblasts. Other markers including fibronectin, N-cadherin and snail, which are expressed through TGF-β1-induced non-smad pathway, are also critical for fibroblasts differentiation into myofibroblasts.

Four members of the histamine-receptor family of G-coupled proteins (H1R, H2R, H3R, and H4R) act as receptors for histamine to mediate various cellular functions, including smooth muscle contraction, changes in blood pressure, gastric acid secretion, and neurotransmitter release [16–18]. In the skin, the pathological processes of pruritus, flush, and urticaria are elicited by activation of histamine-H1R and/or histamine-H2R signaling [19]. Histamine also has distinct effects on skin fibrosis that are characterized by increased levels of glycosaminoglycans [20] and augmentation of fibrogenic cytokine-induced fibroblast proliferation [21]. However, the effects of histamine on αSMA expression remain unknown.

In the present study, we show that histamine-H1R signaling inhibits phosphorylation of Smad2 in skin fibroblasts treated with TGF-β1, subsequently resulting in the suppression of αSMA expression induced by TGF-β/Smad signaling.

2. Materials and methods

2.1. Cell culture

Human primary skin fibroblasts were purchased from Lonza (Walkersville, MD). Human triple-negative breast carcinoma MDA-MB-231 cells were kindly provided by the Minami laboratory. Peripheral blood mononuclear cells (PBMCs) were prepared as described previously [22]. Skin fibroblasts and MDA-MB-231, and PBMC were maintained in Dulbecco's modified Eagle medium (DMEM, Wako Pure Chemicals, Osaka, Japan) and Roswell Park Memorial Institute (RPMI)-1640 (Wako), respectively, containing 10% fetal bovine serum (Tissue Culture Biological, Tulare, CA) and antibiotics (penicillin and streptomycin, Life Technologies, Carlsbad, CA). The skin fibroblasts were used from passage 4–6 in this study.

2.2. Small interfering RNA (siRNA)

Silencer (R) Select Pre-designed siRNAs against *H1R*, s6902 (5′-GUAUCUGGGUU- GCACAUGAtt-3′) and s6904 (5′-UCCU-GUGCAUUGAUCGCUAtt-3′) were purchased from Life Technologies. Non-targeting control siRNA (Low GC, 12935-200) was obtained from Invitrogen. Skin fibroblasts were cultured in 6-well plate with DMEM containing 10% fetal bovine serum. Transfection of skin fibroblasts with 30 pmol siRNA was carried out using lipofectamine RNAiMAX reagent according to the manufacturer's instructions. Fresh medium was added 24 h after transfection, then experiments were conducted for 72 h.

2.3. Real-time polymerase chain reaction (PCR)

Total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) and then subjected to treatment with RNase-free DNase I (Qiagen). For real-time PCR, total RNA (100 ng) was reverse-transcribed using SuperScript®VILO™ master mix (Life Technologies), and real-time PCR was performed on a StepOne Plus system (Applied BioSystems) using TaqMan®Fast Universal PCR master mix (Applied BioSystems) as described previously [23,24]. The TaqMan® Gene Expression Assay (Applied BioSystems) primer/probe pairs used in this study were as follows: Histamine H1 receptor (*HRH1*, Hs00911670_sl), Histamine H2 receptor (*HRH2*, Hs00254569_sl), Histamine H4 receptor (*HRH4*, Hs00222094_ml), αSMA (*ACTA2*, Hs00426835_g1), type I collagen (COL2A1, Hs00264051_ml), fibronectin (*FN1*, Hs00365052_m1), N-cadherin (*CDH2*, Hs00983056_m1), Snail (*SNAI1*, Hs00195591_m1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs99999905_m1). The *HRH1*, *HRH2*, *HRH4*, and *αSMA* mRNA levels were expressed relative to that of *GAPDH* mRNA.

2.4. Antibodies and reagents

The following antibodies were purchased and used for immunoblotting: mouse monoclonal antibodies against αSMA (A2547, Sigma–Aldrich, St. Louis, MO) and β-actin (A1978, Sigma–Aldrich), rabbit monoclonal antibodies against histamine H2 receptor (H2R) (C138612, LifeSpan Biosciences, Seattle, WA), Smad2 (5339, Cell Signaling Technology, Beverly, MA), phospho-Smad2 (3108, Cell Signaling Technology), Smad3 (9523, Cell Signaling Technology), and phospho-Smad3 (9520, Cell Signaling Technology); rabbit polyclonal antibodies against H1R (sc-20633, Santa Cruz Biotechnology, Santa Cruz, CA) and H4R (sc-50312, Santa Cruz Biotechnology). Anti-mouse and anti-rabbit secondary antibodies were obtained from GE Healthcare. Histamine dihydrochloride was purchased from Nacalai Tesque. Recombinant human TGF-β1 was purchased from Wako. Leukotriene C4 was purchased from Funakoshi. Fexofenadine hydrochloride (H1R antagonist) and ranitidine hydrochloride (H2R antagonist) were purchased from Tocris. JNJ7777120 (H4R antagonist) was purchased from Sigma–Aldrich.

2.5. Immunoblot analysis

Whole-cell lysates (WCLs) were prepared from skin fibroblasts and subjected to immunoblotting as described previously [25]. Briefly, the respective cells were solubilized with lysis buffer [50 mM Tris–HCl (pH 7.4), 0.5% (v/v) Nonidet P-40 (Nonidet P-40), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin], and WCLs were prepared by centrifugation at 12,000 × *g* for 20 min at 4 °C. Proteins from WCLs were separated by SDS-PAGE (10% Tris-Glycine gel, Novex, Carlsbad, CA) and transferred onto nitrocellulose (Protran BA83; GE Healthcare). The membranes were immunoblotted with the appropriate antibodies, and bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit IgGs (GE Healthcare) using chemiluminescence reagents (ECL™ prime western blotting detection reagent; GE Healthcare).

2.6. Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD). Differences between two groups were tested for statistical significance by Student's unpaired two-tailed *t*-test or Dunnett's multiple comparison test. A P value <0.05 was considered significant. Statistical analyses were performed using SPSS statistical software (version 21.0; SPSS, Inc., Chicago, IL).

3. Results

3.1. Histamine suppresses TGF-β1-induced αSMA expression in skin fibroblasts

We first assessed the effects of TGF-β1 on the expression of αSMA, a marker of myofibroblast differentiation, as well as type I collagen, fibronectin, N-cadherin and snail in skin fibroblasts. The addition of TGF-β1 to the cells markedly induced the expression of *snail* within 6 h and the expression of *αSMA*, *type I collagen*,

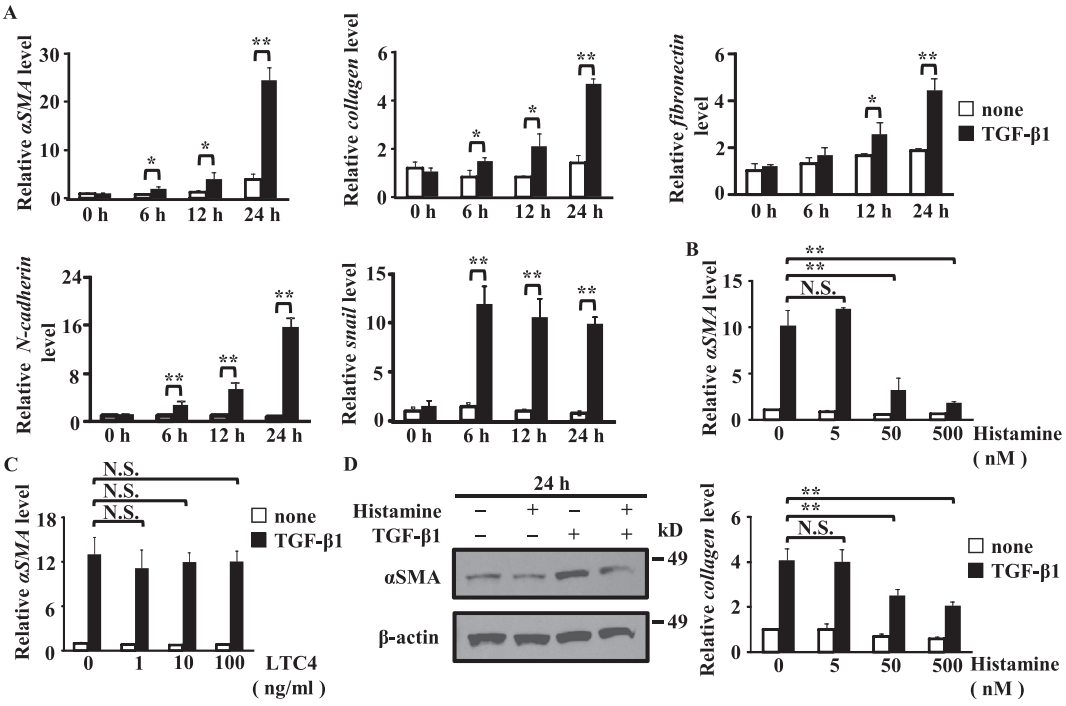

Fig. 1. Histamine inhibits αSMA expression in skin fibroblasts treated with TGF-β1. (**A**–**C**) Human skin fibroblasts were serum-starved for 24 h and then treated with either TGF-β1 (10 ng/mL) or vehicle alone for 24 h in the absence (**A**) or presence of histamine (**B**), or leukotriene C4 (LTC4) (**C**). Subsequently, total RNAs from the respective cells were isolated and subjected to real-time PCR to analyze the mRNA expression levels of *αSMA, type I collagen, fibronectin*, *N-cadherin*, *snail* and *GAPDH*. The amounts of *αSMA*, *type I collagen*, *fibronectin*, *N-cadherin* or *snail* transcripts were expressed relative to that of *GAPDH* transcript. Data are mean ± SD values of three independent experiments. N.S.: not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, by Student's *t*-test (**A**). $^{**}P < 0.01$ vs. without histamine (**B**) or without LTC4 (**C**), by Dunnett's multiple comparison test. (**D**) Human skin fibroblasts were serum-starved for 24 h and then treated with either TGF-β1 (10 ng/mL) or vehicle alone for 24 h in the presence or absence of histamine (500 nM). Subsequently, whole-cell lysates were prepared and analyzed by immunoblotting with αSMA and β-actin as described in Materials and methods. Data are representative of three independent experiments with similar results.

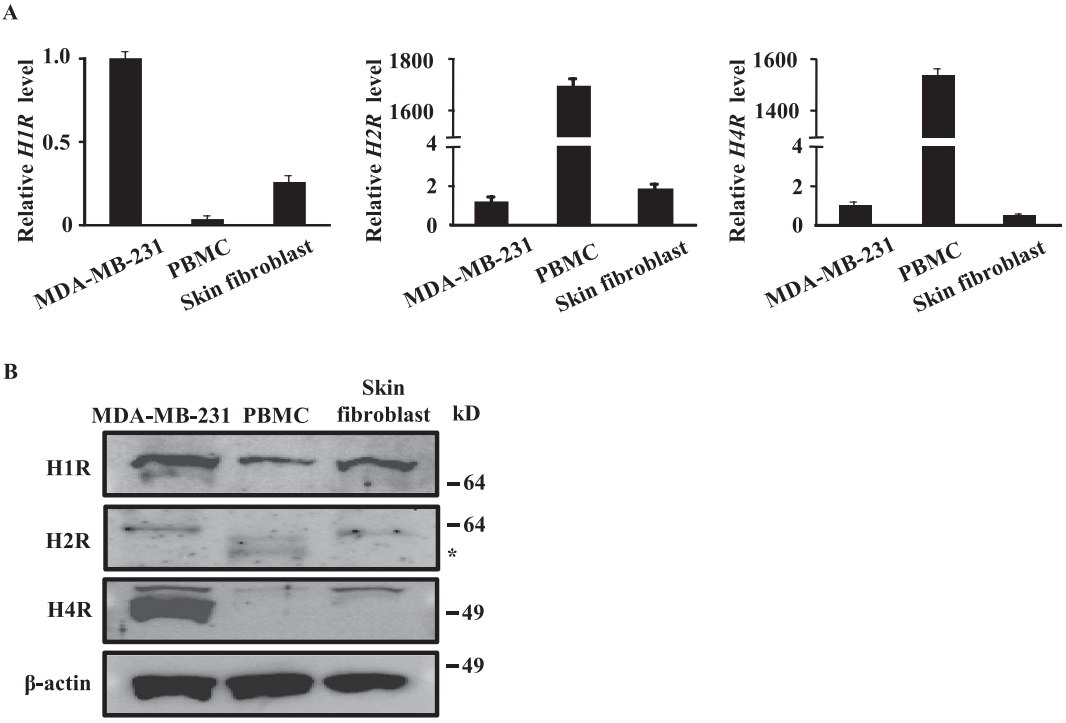

Fig. 2. H1R, but not H2R or H4R, is expressed in skin fibroblasts. (A) Total RNA was isolated from MDA-MB-231, PBMC, and skin fibroblasts, and then subjected to real-time PCR to analyze the mRNA expression levels of *H1R*, *H2R*, *H4R*, and *GAPDH*. The amounts of *H1R*, *H2R*, or *H4R* transcripts were expressed relative to that of *GAPDH* transcript. (**B**) Whole-cell lysates were prepared from MDA-MB-231, PBMC, and skin fibroblasts, and analyzed by immunoblotting with the indicated antibodies, as described in Materials and methods. Data are representative of three independent experiments with similar results. Asterisk (*): band for H2R.

fibronectin, and *N-cadherin* in time-dependent manners, reaching maximum mRNA levels within 24 h (Fig. 1A). However, histamine suppressed the mRNA levels of *αSMA* and *type I collagen* induced by TGF-β1 for 24 h in concentration-dependent manners (Fig. 1B). In contrast, leukotriene C4, a major mediator secreted by mast cells, failed to change the TGF-β1-stimulated expression of *αSMA* (Fig. 1C) and *type I collagen* (data not shown). TGF-β1 also increased αSMA protein levels in skin fibroblasts, and this effect was inhibited by histamine within 24 h (Fig. 1D).

3.2. Expression of histamine receptor, H1R, on skin fibroblasts

We next assessed the expression of the histamine-receptor family of G-coupled proteins on skin fibroblasts using real-time PCR analysis. Skin fibroblasts and MDA-MB-231, a breast cancer cell line used as a positive control, but not PBMC, expressed *H1R* mRNA (Fig. 2A). In contrast, PBMC highly expressed *H2R* and *H4R* mRNA, but only H2R in protein level (Fig. 2A and B), whereas skin fibroblasts expressed neither H2R nor H4R (Fig. 2B). H4R expression wasn't associated between mRNA and protein level in both MDA-MB-231 and PBMC (Fig. 2A and B), possibly due to post-transcriptional gene silencing in histamine receptor expression. Furthermore, skin fibroblasts did not express H3R (data not shown). Thus, skin fibroblasts exclusively expressed H1R among the histamine-receptor family.

3.3. Importance of histamine-H1R for downregulation of αSMA in skin fibroblasts

Next, we assessed the role of H1R in the expression of αSMA in skin fibroblasts. These cells were treated for 30 min with or without histamine receptor antagonists H1RA fexofenadine, H2RA ranitidine, or H4RA JNJ7777120. After stimulation with histamine and TGF-β1 for 24 h, *αSMA* mRNA expression was assessed by real-time PCR. Although TGF-β1 markedly induced *αSMA* expression in skin fibroblasts, histamine inhibited *αSMA* transcripts. However, treatment with H1RA, but not H2RA or H4RA, abrogated the histamine-induced downregulation of *αSMA* mRNA and protein (Fig. 3A and B). In addition, *αSMA* mRNA expression by TGF-β1 was rescued in H1R-knockdowned cells compared to *control* siRNA in the presence of histamine (Fig. 3C). This finding indicates that histamine-mediated signaling via H1R, but not the cognate receptor H2R, contributes to the suppression of αSMA.

3.4. TGF-β1 reduces H1R on skin fibroblasts in a time-dependent manner

We next assessed H1R expression on skin fibroblasts stimulated for up to 48 h with TGF-β1. TGF-β1 significantly decreased *H1R* mRNA expression in skin fibroblasts in a time-dependent manner (Fig. 3D). The H1R protein levels were also reduced in the

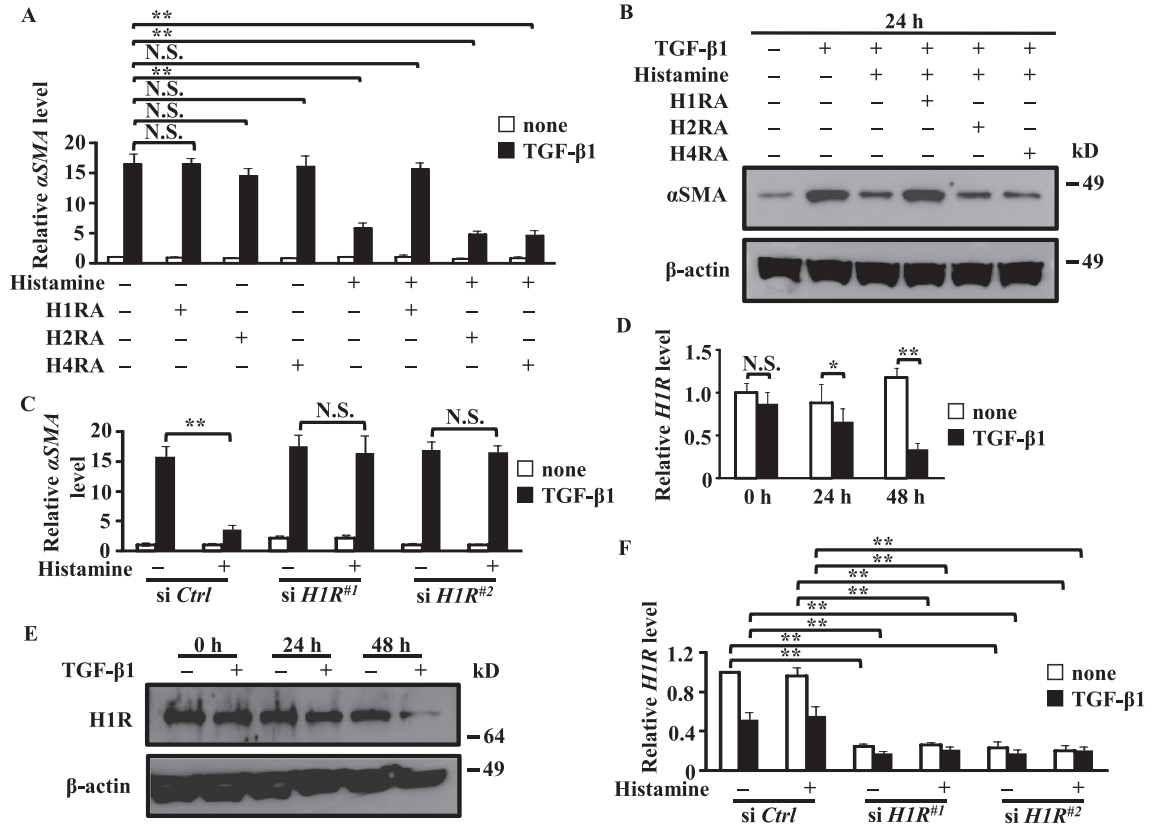

Fig. 3. H1R is crucial for reduced expression of αSMA in skin fibroblasts. (**A**, **B**) Skin fibroblasts were serum-starved for 24 h and further cultured with fexofenadine (H1RA, 10 mM), ranitidine (H2RA, 30 mM), or JNJ7777120 (H4RA, 10 mM) and/or histamine (500 nM) for 30 min, and then stimulated with either TGF-β1 (10 ng/mL) or vehicle alone. (**D, E**) Skin fibroblasts were serum-starved for 24 h and treated with either TGF-β1 (10 ng/ml) or vehicle alone for the indicated time intervals. (**C, F**) Skin fibroblasts were cultured with two kinds of *H1R* siRNA or *control* siRNA, serum-starved for 24 h, further cultured with histamine (500 nM) for 30 min, and then stimulated with either TGF-β1 (10 ng/mL) or vehicle alone for 24 h in turn. (**A, C, D, F**) Total RNA from respective cells was isolated and subjected to real-time PCR to analyze the mRNA expression levels of *αSMA* (**A, C**), *H1R* (**D, F**), and *GAPDH*. The amounts of *αSMA and H1R* transcripts were expressed relative to that of *GAPDH* transcript. Data are mean ± SD values of three independent experiments. N.S.: not significant, $^{**}P < 0.01$ vs. without histamine, by Dunnett's multiple comparison test (**A, F**). $^{*}P < 0.05$, $^{**}P < 0.01$, by Student's *t*-test (**C, D**). (**B, E**) Whole-cell lysates from respective cells were prepared and analyzed by immunoblotting with the indicated antibodies as described in Materials and methods. Data are representative of three independent experiments with similar results. Si *Ctrl*, *control* siRNA; si *H1R*, *H1R* siRNA.

stimulated cells over the 48-h culture period (Fig. 3E). Both *H1R* siRNAs suppressed expression of *H1R* in skin fibroblasts (Fig. 3F).

3.5. Histamine inhibits phosphorylation of Smad2 by TGF-β1 in skin fibroblasts

Finally, to clarify signaling mechanisms induced by TGF-β1, we assessed the expression and phosphorylation of Smad2 and Smad3 on skin fibroblasts by immunoblotting. Although the expression of total Smad2 and Smad3 was comparable before and after stimulation with TGF-β1, TGF-β1 markedly upregulated phosphorylated Smad2 and Smad3 in skin fibroblasts. However, the TGF-β1-mediated phosphorylation of Smad2, but not Smad3, was inhibited by histamine (Fig. 4). These results indicate that histamine/H1R-mediated signaling targets Smad2 to reduce *αSMA* expression induced by TGF-β1 in skin fibroblasts.

4. Discussion

Neutrophils and mast cells accumulate locally during the acute phase of inflammation, while lymphocytes and macrophages are activated during chronic inflammation and the fibrotic phase [26]. Although the involvement of mast cells in skin fibrosis is well known, the roles of histamine and TGF-β1 secreted by mast cells in the pathological processes of skin fibrosis in SSc remain elusive. Here we showed that incubation of skin fibroblasts with histamine suppressed the expression of αSMA induced by TGF-β1 at the protein and mRNA levels, suggesting that histamine acts as a negative regulator of skin fibrosis. We also found that activation of H1R, expressed in skin fibroblasts, suppressed αSMA expression in the cells treated with TGF-β1 and that histamine attenuated TGF-β1 signaling by targeting Smad2. Interestingly, TGF-β1 reduced the expression of H1R itself, resulting in failure of the histamine-mediated effect of suppressing αSMA expression.

Our study indicated that in the early phase of fibrosis, histamine-H1R signaling antagonizes TGF-β1-mediated αSMA expression in skin fibroblast. Histamine, which is produced by mast cells, thus suppresses ECM deposition via decreased expression of type I collagen in the shorter term, whereas histamine itself promotes acute inflammation by inhibiting anti-inflammatory activity of TGF-β1. This indicates that expression of H1R in the shorter term may able to delay the onset of skin fibrosis. Even though, the late phase of fibrosis, histamine fails to suppress ECM expression by consistent activation of TGF-β1 due to downregulation of H1R. Indeed, long-term co-culture of skin fibroblasts with mast cells generates myofibroblasts [27]. Further, once mast cell granules are exhausted [28], expression of ECM can be enhanced due to less histamine, leading to progression of fibrosis. Thus, dynamic regulation between histamine and TGF-β1 by mast cells seems to play an important role in the fibrotic process. It would therefore be interesting to examine the expression of H1R in affected skin tissues from SSc patients or mouse model of graft-versus-host disease, with the potential to delay skin fibrosis if the mechanisms of H1R induction in the local fibroblasts could be clarified.

The present study also highlighted a novel crosstalk between histamine and TGF-β1 signaling in skin fibroblasts. TGF-β1 signaling targets the *αSMA* and *type I collagen* genes [29], suggesting critical roles of such crosstalk in skin fibrosis. *Snail* has also been identified as a TGF-β1-target gene, further implicating the crosstalk between histamine and TGF-β1 via Smad2 in epithelial–mesenchymal transition. Similarly, Smad2 is also implicated in renal fibrosis [30], thus histamine signaling could also be crucial in potentially delaying several types of tissue fibrosis including that of the skin.

It is critical to consider the balance between histamine and TGF-β1 levels in the fibrosis process, and to this end, to study whether skin fibrosis is caused by enhanced TGF-β1 signaling in a histamine-independent manner following decreased histamine or increased TGF-β1 signaling. Clearly, further studies are needed to clarify the mechanism underlying skin fibrosis.

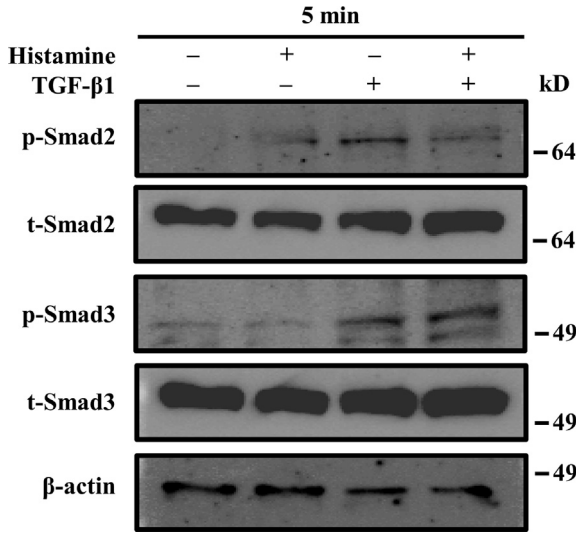

Fig. 4. Histamine inhibits phosphorylation of Smad2 by TGF-β1 in skin fibroblasts. Skin fibroblasts were serum-starved for 48 h and treated with either histamine (500 nM) or vehicle alone for 20 min, and then stimulated with either TGF-β1 (10 ng/mL) for 5 min or vehicle alone. Subsequently, whole-cell lysates were analyzed by the indicated antibodies as described in the Materials and methods section. Data are representative of three independent experiments with similar results.

Conflict of interest

None.

Acknowledgments

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