

Human Eosinophils Have an Intact Smad Signaling Pathway Leading to a Major Transforming Growth Factor- β Target Gene Expression

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Key Words

Eosinophil apoptosis • Transforming growth factor- β • Smad

Abstract

Background: There is a paradoxical finding that eosinophils are frequently accumulated at the sites of allergic inflammation where transforming growth factor (TGF)- β , a negative regulator of eosinophil survival, is upregulated; however, eosinophil accumulation is persistent. We thus hypothesized that eosinophils might have aberrant TGF- β signaling and be unresponsive to TGF- β . To test the hypothesis, we examined the expression and function of Smad proteins, which are central mediators for TGF- β signaling, in human eosinophils. **Methods:** Eosinophils were isolated from the peripheral blood of normal donors, and the expression and activation of endogenous Smad proteins were examined by reverse transcription polymerase chain reaction and Western blotting. The Smad function in the transcription of the major TGF- β target gene Smad7 was investigated using a dominant negative form of Smad3. The effect of TGF- β on eosinophil survival was then evaluated by a cell viability assay using normal and asthmatic eosinophils. **Results:** Human eosinophils expressed mRNAs and proteins of TGF- β

type I and type II receptors, Smad2, Smad3 and Smad4. TGF- β induced the phosphorylation of Smad2 in eosinophils, which was blocked by SB431542, an inhibitor of TGF- β type I receptor kinase. A dominant negative Smad3 protein suppressed TGF- β -induced Smad7 mRNA expression in eosinophils. Finally, TGF- β prevented granulocyte macrophage colony-stimulating factor- or interferon- γ -mediated survival of eosinophils obtained from asthmatic patients as well as normal subjects. **Conclusion:** Human eosinophils have an intact Smad signaling pathway leading to a major TGF- β target gene expression. Thus, eosinophils might become resistant to TGF- β only in vivo circumstances.

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Introduction

Transforming growth factor (TGF)- β is a multifunctional cytokine that is involved in a variety of cellular programs (proliferation, differentiation, extracellular matrix regulation, and survival) [1]. TGF- β principally signals through two different types of serine/threonine kinase receptors, termed type I and type II, and the Smad family of proteins [2–4]. Type I receptor is activated by type II receptor upon ligand binding and induces the

phosphorylation of receptor-activated Smads, Smad2 and Smad3, which in turn form complexes with a common Smad4. The resulting Smad complex then moves into the nucleus, where it regulates the transcription of TGF- β target genes such as Smad7 [5] and c-Jun [6], in cooperation with DNA-binding partners. Smad7 inhibits TGF- β signaling by, in part, competing with Smad2/3 for their binding to the activated type I receptor and is thus a key molecule for negative feedback regulation of TGF- β signaling.

In eosinophils, TGF- β is thought to act as a negative regulator of eosinophil survival. Eosinophils are terminally differentiated, nondividing cells which die of apoptosis in the absence of eosinophil-activating cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-5 [7, 8]. TGF- β abrogates the effects of GM-CSF and IL-5 on the prevention of eosinophil apoptosis [7, 8]. However, paradoxically, TGF- β increases at the sites of allergic inflammation such as in the asthmatic airways and fibrotic tissue [9, 10], where tissue eosinophilia is frequently observed [11] and eosinophil accumulation is also persistent. Furthermore, eosinophils themselves can produce TGF- β in certain situations [9].

These paradoxical observations have prompted us to hypothesize that eosinophils might have aberrant TGF- β signaling, resulting in partial or complete TGF- β resistance. To test the hypothesis, we examined the expression and function of Smad proteins, central intracellular mediators for TGF- β signaling, in human eosinophils. Our results suggest that human eosinophils appear to have an intact TGF- β /Smad signaling pathway, at least in vitro.

Materials and Methods

Reagents

Recombinant human TGF- β_1 , GM-CSF, IL-4, IL-5, IL-13 and interferon (IFN)- γ were purchased from R&D Inc. (Minneapolis, Minn., USA). Poly-IC was purchased from Sigma-Aldrich, Inc. (St. Louis, Mo., USA). A selective inhibitor of TGF- β /activin type I receptor kinase, SB431542 [12], was purchased from Tocris (Ellisville, Mo., USA) and dissolved at a concentration of 10 mM in DMSO as a 1,000 $\times$ stock solution. As SB431542 was dissolved in DMSO, 0.1% DMSO was used as a control for SB431542 in each experiment.

Eosinophil Isolation

Human eosinophils were purified from the peripheral blood obtained from 3 normal volunteer subjects and 3 patients with moderate asthma defined by the criteria of the Japanese Society of Allergology [13]. These patients were not treated with systemic steroids within 4 weeks prior to the eosinophil purification. All

subjects gave written informed consent. Eosinophil purification was performed by Percoll density gradient centrifugation and magnetic cell sorting using MACS anti-CD16 microbeads, as described by Hansel et al. [14]. Briefly, after peripheral blood was overlaid on an isotonic Percoll solution (1.084 g/ml; Sigma-Aldrich, Inc.), the blood was centrifuged at 1,000 g for 30 min at 4°C. Mononuclear cells at the interface were removed, and erythrocytes in the sediment were lysed by two cycles of hypotonic water lysis. Isolated granulocytes were washed twice in phosphate-buffered saline (PBS) supplemented with 2 mM EDTA and 0.5% bovine serum albumin. The cells were then incubated with an equal volume of anti-CD16 monoclonal antibody (mAb) MACS microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 30 min at 4°C with occasional gentle mixing. After 30 min of incubation, the cells were loaded onto the separation column positioned in the MACS (Miltenyi Biotec) magnetic field. The cells were eluted three times with 5 ml PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin. The purity of eosinophils counted by Randolph's stain was >98%.

Cell Culture

Human eosinophils were cultured in RPMI1640 (Gibco/Invitrogen, Carlsbad, Calif., USA) supplemented with 10% fetal calf serum (FCS), 100 μ g/ml streptomycin and 100 U/ml penicillin. Human bronchial epithelial cell line BEAS2B cells [15] were maintained in F-12 Nutrient Mixture medium (Ham) with L-glutamine (Gibco/Invitrogen) supplemented with 10% FCS, 100 μ g/ml streptomycin and 100 U/ml penicillin.

Reverse Transcription Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described [16]. PCR amplification (TGF- β type I and type II receptors, Smad2, Smad3, Smad4 and Smad7 at 94°C for 0.5 min, 57°C for 0.5 min, and 72°C for 1 min, for 40 cycles; histone 3.3 at 94°C for 0.5 min, 48°C for 0.5 min, and 72°C for 1 min, for 40 cycles) was performed in a DNA engine cycler (MJ Research, Inc., Waltham, Mass., USA). The PCR products were separated by 2.0% agarose gel electrophoresis and stained with 0.5 μ g/ml ethidium bromide. The specific primers used are described in table 1. Other primers that are not listed in table 1 were purchased from R&D Inc. and used according to the manufacturer's instructions.

Immunoblotting

Immunoblotting was performed as previously described [16] with specific antibodies for TGF- β type I receptor (Cell Signaling Technology, Beverly, Mass., USA), TGF- β type II receptor (Santa Cruz Biotech, Santa Cruz, Calif., USA), Smad2/3 (Upstate Biotechnology, Lake Placid, N.Y., USA), Smad4 (Santa Cruz Biotech), phosphorylated Smad2 (Upstate Biotechnology), plasminogen activator inhibitor (PAI)-1, and β -actin (Santa Cruz Biotech).

Plasmid Construction and Protein Expression

The Smad3D407E mutant was constructed by replacing wild-type aspartic acid at position 407 with glutamic acid (kindly provided by Dr. Mitsuyasu Kato) [17]. Constructs encoding His₆-tagged non-arginine protein transduction domain (R9-PTD)-containing Smad3D407E (R9/Smad3D407E) were engineered as follows. First, synthesized annealed double-stranded oligodeoxynucleotides (GGATCCAAGCTTGGCAGACGCCGTCGCCGT-

Table 1. RT-PCR primers

Gene	Primer sequence 5'-3'	Size of PCR fragment, bp
TGF- β RII (S) TGF- β RII (As)	AGCAGAAGCTGAGTTCAACCTGGG GGAGCCATGTATCTTGCAGTTCCTCC	702
Smad2 (S) Smad2 (As)	GAATTTGCTGCTCTTCTGGCTCAG GCCATAGGGACCACAACACAATG	322
Smad3 (S) Smad3 (As)	GAGGGCAGGCTTGGGGAAAATG GGGAGGGTGCCGGTGGTGTAAATAC	281
Smad4 (S) Smad4 (As)	AAAGGTGAAGGTGATGTTTGGGTC CTGGAGCTATTCCACCTACTGATCC	268
Smad7 (S) Smad7 (As)	CATCACCTTAGCCGACTCTG GTCTTCTCCTCCCAGTATGC	227
H3.3 (S) H3.3 (As)	CCACTGAACTTCTGATTTCGC GCGTGCTAGCTGGATGTCTT	215

S = Sense; As = antisense; H3.3 = histone 3.3.

CGCCGTCGCCGTGGTAGATCT and its anti-parallel sequence) encoding non-arginine peptide were inserted in frame into the pHA plasmid [18] digested by *Bam*HI (pR9-HA plasmid). Then, complementary DNA (cDNA)-encoding Smad3D407E was inserted in frame into the pR9-HA plasmid digested by *Eco*RI. High-expression BL21 (DE3; Novagen, Madison, Wisc., USA) transformants were selected after blotting lysates of transformants with anti-HA mAb (Covance, Berkeley, Calif., USA). Denatured recombinant R9/Smad3D407E fusion protein was purified by sequential Ni^{2+} nitrilotriacetic acid-agarose chromatography, fast protein liquid ion exchange chromatography and gel filtration chromatography, as previously described [18]. Proteins were stored at -70°C in PBS/10% glycerol and thawed immediately before use. SDS-PAGE was performed with NuPAGE 4–12% Bis-Tris gels and MOPS running buffer (Invitrogen Japan, Tokyo, Japan). Endotoxin contamination of recombinant proteins was determined via a Limulus lysate assay (BioWhittaker, Walkersville, Md., USA). Recombinant proteins were treated with polymyxin B sulfate (50 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) before addition to cell culture medium. Control R9/LACK [19] fusion protein was also generated and purified in a similar manner.

Laser Microscopy

Cy3 donkey anti-mouse immunoglobulin G (IgG) was purchased from Jackson Immuno Research (West Grove, Pa., USA). After incubation with R9/Smad3D407E or R9/LACK protein (150 nM for 2 h), eosinophils were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.2% Triton-X, blocked with 5% donkey serum, and stained with 5 $\mu\text{g}/\text{ml}$ anti-HA mAb and secondary Ab. Stained cells were analyzed using a Personal Laser microscope (Nicon, Tokyo, Japan).

Quantitative Real-Time PCR

Total RNA was extracted from cells (approximately $1\text{--}2 \times 10^6$) using Isogen solution (Nippon Gene, Toyama, Japan), ac-

cording to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using the Reverse Transcriptase System (Applied Biosystems, Foster City, Calif., USA). Quantitative real-time PCR analysis was performed using the ABI7500 real-time PCR system (Applied Biosystems), according to the manufacturer's instructions, using the specific primer and probes purchased from Applied Biosystems. The ratio of each gene to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated, and the value of 1.0 was assigned to eosinophils that were incubated without TGF- β_1 .

ELISA

The amounts of TGF- β_1 in the culture supernatants were determined using the human TGF- β_1 ELISA kit (R&D), according to the manufacturer's instructions. The samples were activated by acidification procedure prior to the ELISA assay for determination of the total TGF- β_1 concentration unless indicated for determination of the active form of TGF- β_1 concentration.

Cell Viability Assay

Cells (2.5×10^4) were cultured with or without 10 ng/ml GM-CSF or IFN- γ in the presence or absence of 10 μM SB431542 or 10 ng/ml TGF- β_1 for 72 h in a 96-well microtiter plate. The number of viable cells in each well was then determined by the WST assay using the Tetra Color One kit (Seikagaku Corporation, Tokyo, Japan), according to the manufacturer's instructions.

Data Analysis

Data are summarized as the mean $\pm$ SD of triplicate samples in each group. A statistical analysis was performed by the unpaired Student's *t* test. $p < 0.05$ was considered significant.

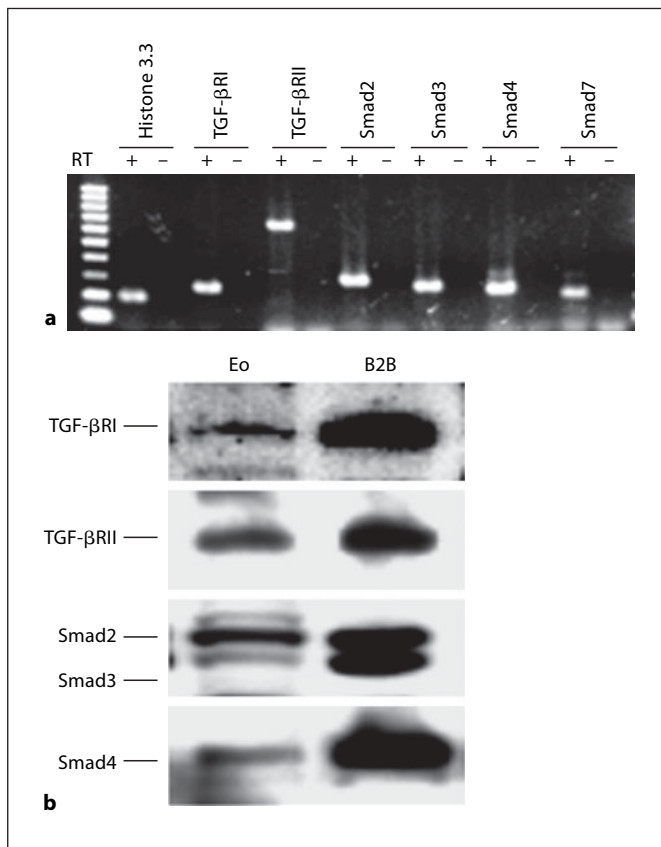


Fig. 1. Expression of TGF- β receptors and Smad proteins in human eosinophils. **a** RNA was extracted from eosinophils isolated from normal donors, and RT-PCR using specific primers for TGF- β type I and II receptors, Smad2, Smad3, Smad4, Smad7 and histone 3.3 was performed. RT (-) = PCR amplification of the original RNA samples without RT to cDNA (negative control). **b** Eosinophil cell lysates were subjected to a Western blot analysis with specific antibodies against TGF- β type I receptor, type II receptor, Smad2/3 and Smad4. BEAS2B human bronchial epithelial cell lines (B2B) are used as a positive control. Representative results of three independent experiments using eosinophils (Eo) from 3 normal donors are shown (**a**, **b**).

Results

mRNA and Protein Expression of TGF- β Receptors and Smads in Human Eosinophils

We first examined the expression of TGF- β receptors and Smad proteins in human eosinophils. An RT-PCR analysis showed that human eosinophils significantly expressed mRNAs of TGF- β type I and type II receptors, Smad2, Smad3, Smad4 and Smad7 (fig. 1a). A Western blot analysis showed that human eosinophils expressed TGF- β type I and type II receptors, Smad2, Smad3 and Smad4 proteins (fig. 1b).

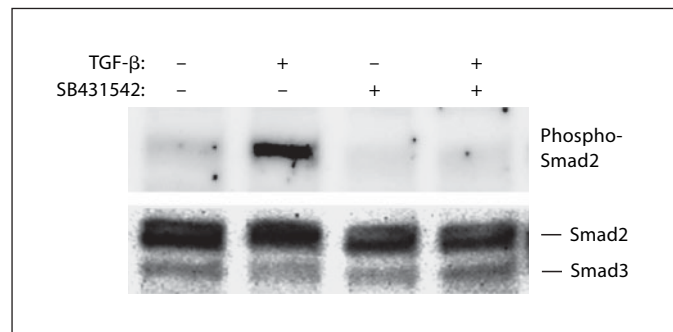


Fig. 2. Human eosinophils express functional TGF- β receptors. Eosinophils were stimulated with 10 ng/ml TGF- β ₁ for 30 min in the presence or absence of 10 μ M SB431542. Cells were then lysed, and a Western blot analysis was performed with specific antibodies against phosphorylated Smad2 (Phospho-Smad2) and Smad2/3. Representative results of three independent experiments using eosinophils from 3 normal donors are shown.

Human Eosinophils Express Functional TGF- β Receptors

To determine whether TGF- β receptors expressed on human eosinophils were functional, we examined the effect of TGF- β on the phosphorylation of Smad2. As shown in figure 2, TGF- β induced phosphorylation of Smad2 in human eosinophils, which was completely blocked with SB431542, a selective inhibitor of TGF- β type I receptor kinase [12]. The results indicated that human eosinophils express functional TGF- β receptors. It should be noted that FCS contains significant amounts of the latent, but not active, forms of TGF- β ₁ and TGF- β ₂ (see below), and the latent forms do not affect activation of TGF- β signaling as indicated in these experiments.

A Dominant Negative Form of Smad3 Suppresses TGF- β -Induced mRNA Expression of Smad7, a Putative Smad Target Gene, in Human Eosinophils

To determine whether the Smad signaling pathway is functional in eosinophils, we examined the effect of a mutant Smad3 protein (Smad3D407E) that acts as a dominant negative form of Smad3 through the inhibition of phosphorylation of Smad3 [17] on mRNA induction of the TGF- β /Smad putative target gene Smad7 [5]. Because eosinophils are normally refractory to standard methods of gene delivery, we used a cell membrane-permeable form of Smad3D407E which fused to poly-arginine (R9)-PTD. R9 peptide can efficiently deliver target proteins into a variety of cells in a receptor-independent manner

[20]. As shown in figure 3a, an immunofluorescence study using anti-HA antibody confirmed that control R9/LACK and R9/Smad3D407E fusion proteins were comparably expressed in eosinophils after their introduction. The introduction of R9/Smad3D407E but not R9/LACK fusion protein inhibited the TGF- β -induced expression of PAI-1, a TGF- β /Smad target gene in epithelial cells [21], in human bronchial epithelial cell line BEAS2B cells (fig. 3b), thus confirming the dominant negative effect of R9/Smad3D407E on TGF- β /Smad signaling. A real-time PCR analysis showed that Smad7 mRNA expression was increased at 30 min after TGF- β stimulation and then gradually decreased in human eosinophils (fig. 3c). The introduction of R9/Smad3D407E fusion protein into eosinophils abolished the Smad7 mRNA induction by TGF- β (fig. 3c).

Human Eosinophils Constitutively Express TGF- β_1 and TGF- β_2 mRNAs, but Not the Proteins in vitro

Previous studies reported that eosinophils produce TGF- β in certain situations [9]. Since we found that human eosinophils possess a functional TGF- β /Smad signaling pathway, we asked whether eosinophils produce TGF- β , and, if so, whether the endogenous TGF- β transduces its signaling in an autocrine manner. We first examined the expression of TGF- β_1 and TGF- β_2 mRNA in human eosinophils by conventional and real-time RT-PCR. We found that eosinophils constitutively expressed TGF- β_1 and TGF- β_2 mRNAs (fig. 4a). TGF- β_1 significantly enhanced the TGF- β_1 mRNA expression, whereas IL-4, IL-13 and Poly-IC did not affect the TGF- β_1 mRNA expression (fig. 4b). The induction of TGF- β_2 mRNA by TGF- β_1 was also observed in eosinophils (data not shown). Although eosinophils constitutively expressed TGF- β_1 and TGF- β_2 mRNAs, an ELISA assay showed that eosinophils did not produce TGF- β_1 protein regardless of the stimulations with IL-4, IL-5, GM-CSF or IFN- γ (fig. 4c). We could neither detect spontaneous nor IL-4-, IL-5-, GM-CSF- or IFN- γ -induced TGF- β_2 protein production in human eosinophils (data not shown). In addition, we could not detect the active forms of TGF- β_1 and TGF- β_2 proteins in any samples examined (data not shown). These results suggest that eosinophils do not produce TGF- β in the basal and eosinophil-activating conditions in vitro. It should be noted that 10% FCS contained significant amounts of the latent forms of TGF- β_1 and TGF- β_2 , but did not contain the active forms (fig. 4c; data not shown).

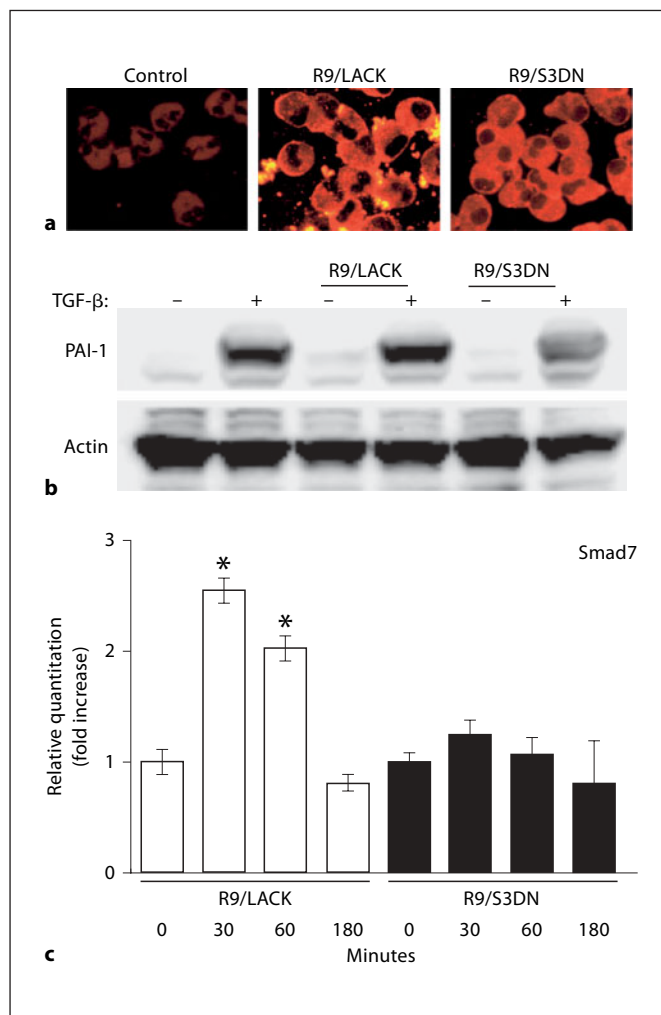
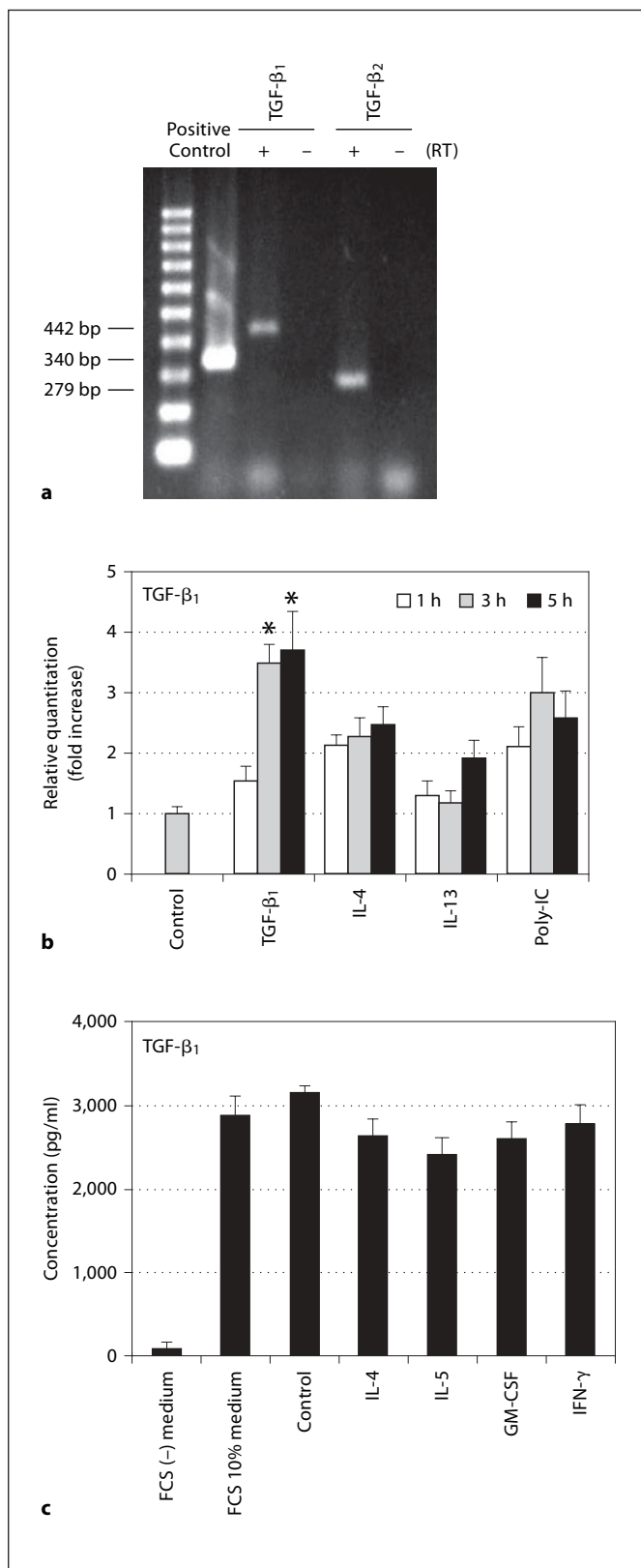


Fig. 3. Dominant negative form of Smad3 suppresses TGF- β -induced mRNA expression of Smad7, a putative Smad target gene, in human eosinophils. **a** Distribution of R9/LACK and R9/Smad3D407E fusion proteins in human eosinophils. Eosinophils were incubated with R9/LACK or R9/Smad3D407E fusion protein (150 nM each) for 2 h, fixed, permeabilized, stained with anti-HA mAb and Cy3 donkey anti-mouse IgG, and analyzed via laser microscopy. Positive staining is indicated as red. **b** Introduction of R9/Smad3D407E fusion protein inhibited the TGF- β -induced expression of PAI-1 in human bronchial epithelial cell line BEAS2B cells. Cells were preincubated either with 150 nM of R9/LACK or R9/Smad3D407E fusion protein and then stimulated with 10 ng/ml TGF- β_1 for 24 h. The total cell extracts were prepared and analyzed with specific antibodies against PAI-1 and β -actin by an immunoblot analysis. **c** Eosinophils were preincubated either with 150 nM of R9/LACK or R9/Smad3D407E fusion protein and then treated with 10 ng/ml TGF- β_1 for indicated times. RNA was extracted, and real-time PCR using the specific primers and probe for Smad7 and GAPDH was performed. Representative results of three independent experiments using eosinophils from 3 normal donors are shown. * $p < 0.05$ in comparison with the corresponding control (unpaired Student's t test).



TGF-β Prevents GM-CSF- or IFN-γ-Mediated Survival of Eosinophils Derived from Asthmatic Patients and Normal Subjects

Finally, we examined the effect of TGF-β on GM-CSF- or IFN-γ-mediated prevention of eosinophil apoptosis (fig. 5). The addition of GM-CSF to the eosinophil culture prolonged eosinophil survival as previously reported (fig. 5a) [7, 8]. A dose of 10 μM SB431542, which was optimal for inhibition of Smad2 phosphorylation (fig. 2), did not affect the GM-CSF-mediated eosinophil survival (fig. 5a). In contrast, exogenously added TGF-β did inhibit, although only partially, the GM-CSF- and IFN-γ-mediated eosinophil survival (fig. 5b), as previously reported [7, 8]. We also compared the responsiveness to TGF-β between eosinophils obtained from asthmatics and from normal subjects (fig. 5c). We found that TGF-β inhibited the GM-CSF- and IFN-γ-mediated survival of eosinophils derived from moderate asthmatic patients as well as from normal subjects. These results suggest that there are no differences in responsiveness to TGF-β between asthmatic and normal eosinophils, at least in terms of in vitro survival.

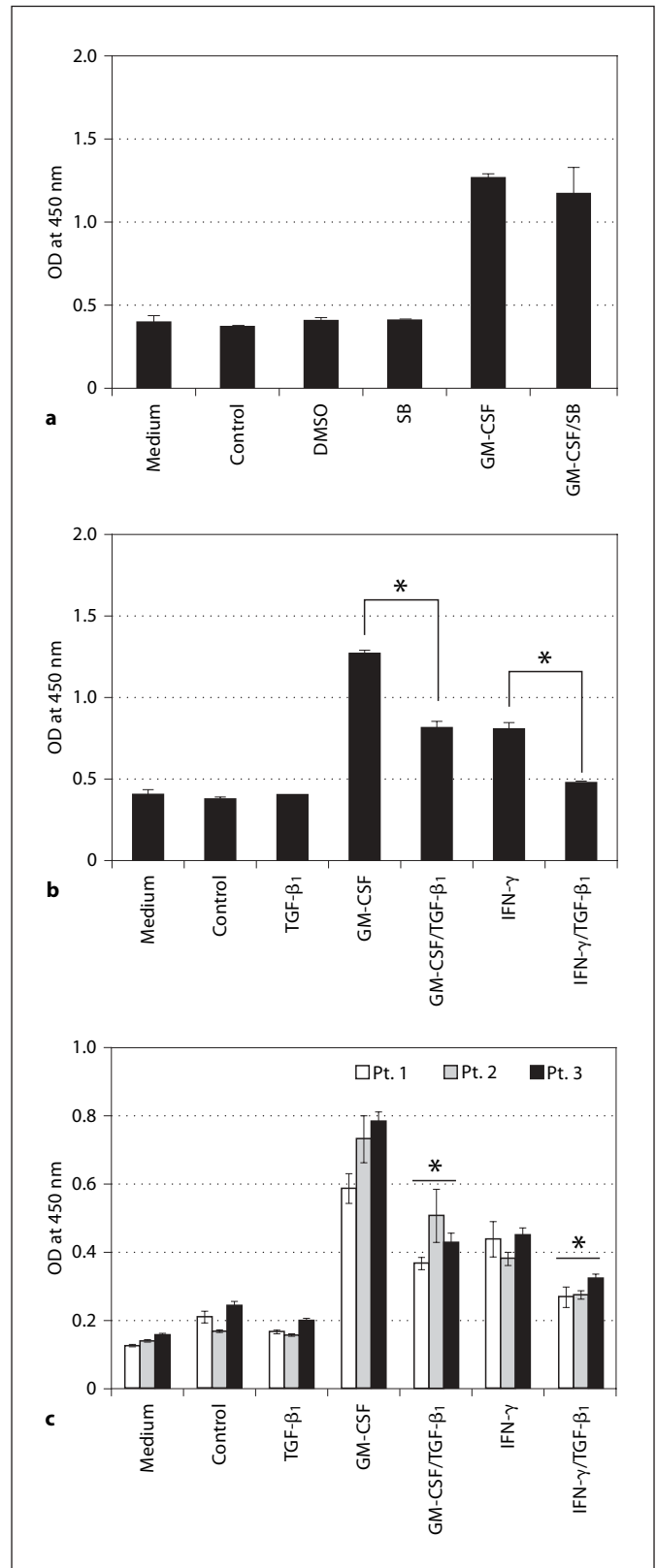
Fig. 4. Human eosinophils constitutively express TGF-β₁ and TGF-β₂ mRNAs, but not the proteins. **a** RNA was extracted from eosinophils isolated from normal donors, and RT-PCR was performed using specific primers for TGF-β₁, TGF-β₂ and histone 3.3. RT (-) = PCR amplification of the original RNA samples without RT to cDNA (negative control). **b** Eosinophils were stimulated with 10 ng/ml TGF-β₁, IL-4, IL-13 or Poly-IC for the indicated times. RNA was then extracted from the eosinophils, and real-time PCR was performed using the specific primers and probes for TGF-β₁ and GAPDH. The ratio of each gene to that of GAPDH was calculated, and the value of 1.0 was assigned to eosinophils that were incubated without TGF-β₁. The values represent the mean ± SD of triplicate samples per group. * p < 0.05 in comparison with the corresponding control (unpaired Student's t test). Representative results of three independent experiments using eosinophils from 3 normal donors are shown. Control = No stimulation. **c** Eosinophils were cultured in 10% FCS-containing medium in the presence or absence of 10 ng/ml IL-4, IL-5, GM-CSF or IFN-γ for 48 h. Culture supernatants were then collected, and the concentrations of total TGF-β₁ were estimated by ELISA. The values represent the mean ± SD of triplicate samples per group. FCS (-) medium = FCS-free medium alone; FCS 10% medium = medium containing 10% FCS alone; control = cells without any stimulation. Representative results of three independent experiments using eosinophils from 3 normal donors are shown.

Discussion

Eosinophils are terminally differentiated, nondividing cells that are refractory to gene introduction and present approximately 1–5% in human peripheral blood [22]. These features of eosinophils have made it somewhat difficult to study the intracellular mechanisms underlying their variety of biological responses. Fujihara et al. [23] recently reported that I κ B α fused to an 11 amino acid HIV-Tat peptide PTD was taken up by human eosinophils in a receptor-independent manner and successfully functioned as a selective inhibitor of nuclear factor- κ B. Taken together with our findings using R9-PTD domain fusion Smad3D407E protein (fig. 3), the direct delivery of proteins with PTD may thus be a useful tool for a detailed molecular analysis of the signaling pathways in eosinophils that are normally refractory to standard methods of gene delivery.

Although eosinophils expressed TGF- β_1 and TGF- β_2 mRNAs (fig. 4a), a selective inhibitor of TGF- β type I kinase activity SB431542 did not affect GM-CSF-mediated prevention of eosinophil apoptosis (fig. 5a), thus suggesting that there were no effects of endogenous TGF- β on eosinophil survival in an autocrine manner. Consistent with these findings, we could not detect any significant amount of TGF- β_1 protein both at the basal and eosinophil-activating conditions (fig. 4c). Thus, although eo-

Fig. 5. Exogenous, but not endogenous, TGF- β prevents GM-CSF or IFN- γ -mediated eosinophil survival. **a** Eosinophils (2.5×10^4) were cultured in the presence or absence of 10 ng/ml GM-CSF with or without 10 μ M SB431542 for 72 h. Cellular viability was then estimated by a WST assay. Representative results of three independent experiments using eosinophils from 3 normal donors are shown. **b** Eosinophils (2.5×10^4) were cultured in the presence or absence of 10 ng/ml GM-CSF or IFN- γ with or without 10 ng/ml TGF- β_1 for 72 h. Cellular viability was then estimated by a WST assay. Representative results of three independent experiments using eosinophils from 3 normal donors are shown. **c** Eosinophils (2.5×10^4) obtained from 3 moderate asthmatics, patients 1–3 (Pt. 1, Pt. 2, Pt. 3), were cultured in the presence or absence of 10 ng/ml GM-CSF or IFN- γ with or without 10 ng/ml TGF- β_1 for 72 h. Cellular viability was then estimated by a WST assay. **a–c** Data are represented as OD₄₅₀. Values represent the mean $\pm$ SD of triplicate samples per group. * $p < 0.05$ in comparison with the corresponding control (unpaired Student's t test). Medium = Medium alone; control = cells without stimulation; DMSO = cells stimulated with control vehicle DMSO.



sinophils express TGF- β_1 and TGF- β_2 mRNAs at the basal condition, we speculate that the mRNAs might not be significantly translated into the proteins, or TGF- β_1 and TGF- β_2 proteins might be rapidly degraded.

Our results suggest that eosinophils possess intact signaling components of TGF- β , and normally, are able to respond to TGF- β , at least in vitro. Our results also suggest that there are no differences in responsiveness to TGF- β between asthmatic and normal eosinophils, at least in an in vitro survival assay. Therefore, we speculate that eosinophils might become resistant to TGF- β only in in vivo circumstances. Since eosinophils are exposed to a milieu of inflammatory or fibrogenic mediators that can activate eosinophils at the disease sites including secretory IgA, platelet-activating factor and cytokines (e.g., IL-5, IFN- γ , GM-CSF) [22], these signaling pathways might negatively regulate TGF- β signaling in eosinophils in vivo. In fact, it was reported that IFN- γ inhibited TGF- β /Smad signaling by inducing inhibitory Smad7 in certain cell lines [24], although we did not find the effects of IFN- γ on the induction of Smad7 mRNA in human eosinophils and the TGF- β inhibition of eosinophil survival (fig. 5; data not shown).

Not only TGF- β but also bone morphogenetic proteins and activins, members of the TGF- β superfamily, are upregulated in asthmatic airways, and these cytokines can induce Smad7 [25]. Thus, they might also be candidates acting in vivo to counteract TGF- β signaling through the upregulation of Smad7. We are currently investigating this possibility.

Recent studies reported that a majority of the cells infiltrating into the airways in mouse asthma models ex-

pressed phosphorylated Smad2, thus suggesting that they undergo TGF- β signaling in vivo [26, 27]. It remains unclear which inflammatory cell types express phosphorylated Smad2. However, since eosinophils are major cells infiltrating into asthmatic airways, the phosphorylated Smad2-positive cells appear to be eosinophils. These findings may suggest an alternative possibility that, although eosinophils undergo apoptosis at the site of the TGF- β -rich microenvironment, the number of eosinophils recruited to the site might exceed the number of cell death and, as a result, eosinophils might appear to be accumulated in vivo.

In summary, we showed that eosinophils possess intact Smad signaling components of TGF- β and normally respond to TGF- β , at least in vitro. To our knowledge, this is the first demonstration revealing the presence and function of the TGF- β /Smad signaling pathway in eosinophils. Our current information will be important in asthma or allergy research given the increasing interest in the role of TGF- β in eosinophils in terms of the development of airway remodeling in asthma [9].

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