

T Cell Immunoglobulin Mucin Domain (TIM)-3 Promoter Activity in a Human Mast Cell Line

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T cell immunoglobulin mucin domain (TIM)-3 is an immunomodulatory molecule and upregulated in T cells by several cytokines. TIM-3 also influences mast cell function but its transcriptional regulation in mast cells has not been clarified. Therefore, we examined the transcript level and the promoter activity of TIM-3 in mast cells. The TIM-3 transcript level was assessed by real-time RT-PCR and promoter activity by luciferase reporter assay. TIM-3 mRNA levels were increased in HMC-1, a human mast cell line by TGF- β 1 stimulation but not by stimulation with interferon (IFN)- α , IFN- λ , TNF- α , or IL-10. TIM-3 promoter -349~+144 bp region relative to the transcription start site was crucial for the basal and TGF- β 1-induced TIM-3 promoter activities in HMC-1 cells. TIM-3 promoter activity was increased by overexpression of Smad2 and Smad4, downstream molecules of TGF- β 1 signaling. Our results localize TIM-3 promoter activity to the region spanning -349 to +144 bp in resting and TGF- β 1 stimulated mast cells.

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INTRODUCTION

T cell immunoglobulin mucin domain (TIM)-3 is expressed in various leukocyte subpopulations and modulates their functions (1). It is expressed on the surfaces of exhausted T cells and involved in the downregulation of effector function of T cells (2-4). TIM-3 on dendritic cells promotes the uptake of apoptotic cells via interaction with phosphatidylserine but suppresses the immunogenicity of nucleic acid via in-

teraction with HMGB1 (5,6). TIM-3 expression in mast cells influences cytokine production and apoptosis of these cells (7). However, the regulation of TIM-3 expression has not been well known.

Mast cells play a significant role in various immune responses. These cells lead allergic symptoms through secretion of mediators and cytokines upon cross-linking of Fc ϵ RI (8). Mast cells contribute defense against pathogen; mice devoid of mast cells die from infection that does not result in death in wild type mice (9). Protective immunity is enhanced by mast cell production of TNF- α and recruitment of neutrophils to the infectious site (9). Mast cells are also required for the development of collagen-induced rheumatoid arthritis and the induction of tolerance to skin graft (10,11).

To understand the regulation of TIM-3 expression in mast cells, we investigated the effect of various cytokines on TIM-3 transcription and the activity of TIM-3 promoter in relation with TGF- β 1 stimulation of mast cells.

MATERIALS AND METHODS

Reagents

IFN- α , IFN- λ , TGF- β 1, IL-10, and TNF- α were purchased from R&D Systems (Minneapolis, MN, USA). Expression vectors for Smad2 and Smad4 were kindly provided by Dr. Cho (Dept. Biochemistry, Ajou University School of Medicine, Korea).

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Abbreviation: TIM-3, T cell immunoglobulin mucin domain-3

Cell culture

HMC-1, a human mast cell line, was kindly provided by Professor Hyung Min, Kim (Kyunghee University, Suwon, Korea). HMC-1 cells were maintained in IMDM supplemented with 10% FBS, penicillin-streptomycin (each 100 U/ml, 100 ug/ml). Similarly, HEK293 cells were maintained in RPMI 1640 (Gibco BRL, Paisley, Scotland) supplemented with 10% FBS.

Real-time RT-PCR

The total RNA was isolated using RNA STAT-60 (Tel-Test, IN C., Friendwood, TX, USA) and reverse transcribed using Superscriptase II (Invitrogen, Carsbade, CA, USA). Real-time PCR was performed using primers (5'-TCCAAGGATGCTTACCACCAG-3': 5'-GCCAATGTGGATATTTGTGTTAGATT-3') and a Taq Man probe (5'-ACATGGCCCAGCAGAGACACAGACACT-3') for TIM-3 transcript which was normalized to GAPDH transcript levels.

Plasmid construction

Luciferase reporter vectors were constructed by ligation of human TIM-3 promoter region DNA fragment into pGL-Basic vector (Promega, Madison, WI, USA). Human TIM-3 promoter DNA was amplified by PCR using genomic DNA isolated from HMC-1 cells. For amplification of TIM-3 -1677 ~ +144 DNA fragment, primers TIM3+1 (5'-GGAGCTTGCAGAAGAAAAGTCAGAGGACACCTCTGTTAGG-3') and 5'-AGAGCCTTGACCAAGTTCATGCTGCTAATAAAAAATAACCCAG-3' were used. For TIM-3 -872 ~ +144 DNA fragment, primers TIM3+1 and 5'-CTTTTGCTTTTAAAGGTGCCAGATAAAGGT-CACACTCCCAG-3' were used. For TIM-3 -349 ~ +144 DNA fragment, primers TIM3+1 and 5'-CTGTGACCAAAGTTTATGAAGCC-3' were used. The PCR products were cloned into Topo TA cloning vector (Invitrogen) and the nucleotide sequences were verified by comparison with the gene sequence (NW_001838954). Then TIM-3 promoter DNA was subcloned into pGL-Basic vector using *Nhe* I and *Bgl* II and designated as T3U(1.8)-luc, T3U(1.0)-luc and T3U(0.5)-luc.

Luciferase reporter assay

HMC-1 cells (1×10^6) were transfected with 3.6 μ g of luciferase reporter vector together with 400 ng of pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) using electroporator (Digital Bio Technology, Seoul, Korea). The cells were incubated for 48 h in 5% CO₂ incubator at the 37°C, and then luciferase activity was analyzed using luciferin

(Promega, Madison, WI, USA) and luminometer (Molecular devices, Sunnyvale, CA, USA). The transfection efficiency was analyzed by GFP-expressing cell frequencies using flow cytometer (FacsCanto). Similarly, luciferase activity was assessed in HEK293 cells transfected with DNA using Lipofectamin 2000 (Invitrogen, Carsbade, CA, USA).

Statistics

Student's t-test (p-value < 0.01) was used to determine statistical significance.

RESULTS

TIM-3 transcription was upregulated in HMC-1 cells by TGF- β 1 stimulation

In recent studies, TIM-3 mRNA and protein expression levels were up regulated in TGF- β 1 stimulated- human mast cells (12) but the transcriptional regulation of TIM-3 by other stimulants has not been well explored. To address this issue a human mast cell line, HMC-1 cells were treated with various cytokines for the indicated time and then TIM-3 mRNA levels were determined by real-time RT-PCR. TIM-3 mRNA expression was not significantly enhanced by treatment with the indicated concentration of IFN- α , IFN- λ , IL-10, or TNF- α (p > 0.05) but was significantly increased by TGF- β 1 stimulation of HMC-1 cells for 4 h (p < 0.005) (Fig. 1).

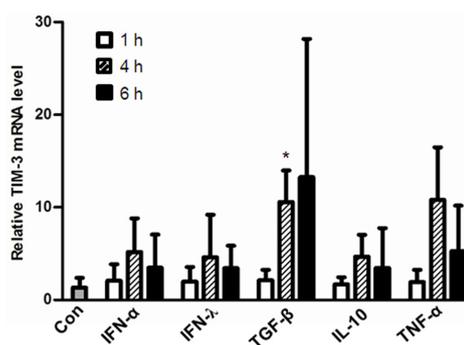


Figure 1. TIM-3 mRNA expression was increased in HMC-1 cells by stimulation with TGF- β 1 but not by stimulation with IFNs, IL-10, and TNF- α . HMC-1 cells were treated for the indicated time with IFN- α (100 U/ml), IFN- λ (100 ng/ml), TGF- β 1 (2 ng/ml), IL-10 (10 ng/ml), and TNF- α (10 ng/ml), respectively and then TIM-3 transcript levels were analyzed by real-time RT-PCR. TIM-3 transcript levels were normalized to GAPDH transcript levels. Relative Tim-3 mRNA levels to control (Con, without cytokine treatment) were presented. *p < 0.005.

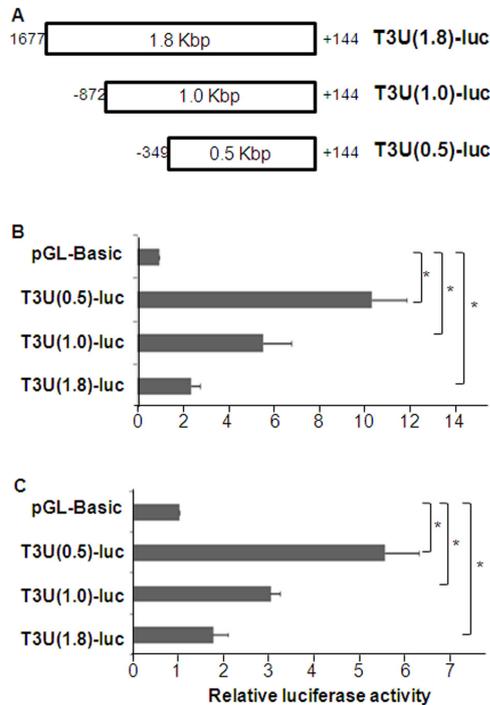


Figure 2. Basal *TIM-3* promoter activity in HEK293 and HMC-1 cells. (A) Luciferase reporter vectors containing various length of *TIM-3* promoter region. (B) HEK293 (5×10^5) or (C) HMC-1 (1×10^6) cells were transfected with the indicated luciferase reporter vector together with pEGFP-N1 vector (1/10 of total plasmid) and 48 h later luciferase activity was measured and normalized to the frequencies of GFP-expressing cells. Relative luciferase activity to control (pGL-Basic) was presented. The data are the mean \pm standard deviation of 5 experiments. * $p < 0.01$.

TIM-3 promoter activity was increased in HMC-1 cells by TGF- β 1 stimulation

To know the *TIM-3* promoter is responsive to TGF- β 1 stimulation, we first examined the basal activity of *TIM-3* promoter by luciferase reporter assay using vectors depicted in Fig. 2A. Luciferase activities in both HEK293 cells and HMC-1 cells transfected with T3U(0.5)-luc that contains proximal *TIM-3* promoter were significantly higher than that of controls transfected with the empty vector pGL-Basic (Fig. 2B and C). Also, luciferase activities driven by T3U(1.0)-luc and T3U(1.8)-luc, respectively were significantly higher than that of controls but lower than that driven by T3U(0.5)-luc. These results indicate that the proximal *TIM-3* promoter spanning from +144 to -349 may consist of sufficient element for the basal level transcription of *TIM-3* in HMC-1 cells.

We next analyzed the *TIM-3* promoter activity in the presence of TGF- β 1 stimulation (Fig. 3). Luciferase activity driv-

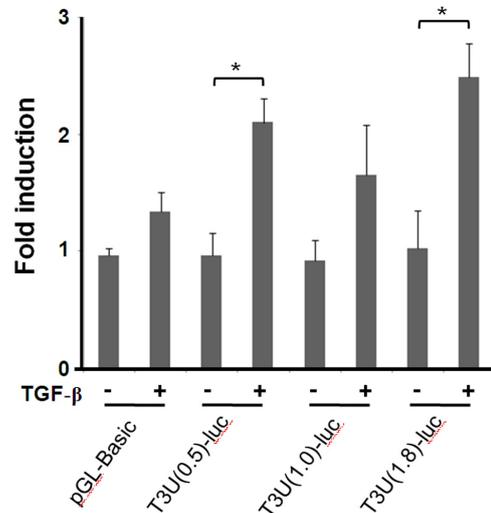


Figure 3. *TIM-3* promoter activity in HMC-1 cells stimulated with TGF- β 1. HMC-1 (1×10^6) cells were transfected with the indicated luciferase reporter vector together with pEGFP-N1 vector (1/10 of total plasmid) and 42 h later treated with TGF- β 1 (2 ng/ml) for 6 h. Then luciferase activity was measured and normalized to the frequencies of GFP-expressing cells. Fold induction of luciferase activity by TGF- β 1 treatment was presented for each luciferase reporter vector. The data are the mean \pm standard deviation of 5 experiments. * $p < 0.001$.

en by each luciferase reporter vector in the presence of TGF- β 1 was presented as fold induction relative to that in the absence of TGF- β 1. Luciferase activity in HMC-1 cells transfected with T3U(0.5)-luc or T3U(1.8)-luc was significantly increased (2.3 and 2.6 fold, respectively) by TGF- β 1 stimulation compared to that in the absence of TGF- β 1 stimulation ($p < 0.001$). However, luciferase activity in HMC-1 cells transfected with T3U(1.0)-luc or the empty pGL-Basic was not significantly increased by TGF- β 1 stimulation compared to that in the absence of TGF- β 1 stimulation ($p > 0.01$). These results suggest that *TIM-3* promoter region may contain the TGF- β 1 responsive elements.

TIM-3 promoter activity was upregulated by Smad2 and Smad4 overexpression

To support the TGF- β 1 responsiveness of the *TIM-3* promoter, we assessed *TIM-3* promoter activity in the cells overexpressing Smad2 and Smad4 that were reported to be involved in the gene expression induced by TGF- β 1 (13,14). Given that TGF- β responsive promoter activity was increased by overexpression of either Smad2 or Smad3 in HEK293 cells even in the absence of TGF- β stimulation (15), we analyzed *TIM-3* promoter activity in HEK293 cells overexpressing

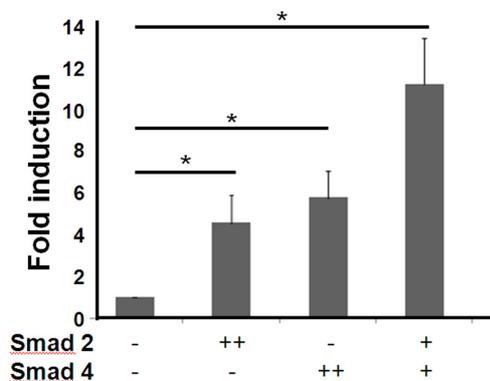


Figure 4. TIM-3 promoter activity was increased by Smad overexpression in HEK293 cells. HEK293 cells (1×10^6) were transfected with T3U(1.8)-luc ($1.8 \mu\text{g}$) together with the indicated Smad expression vector (+: $0.9 \mu\text{g}$, ++: $1.8 \mu\text{g}$) or control empty vector pcDNA ($1.8 \mu\text{g}$). For normalization of transfection efficiency, pEGFP-N1 vector (1/10 of total plasmid) was also co-transfected. After 48 h, the luciferase activity was measured and normalized to the frequencies of GFP-expressing cells. Fold induction of luciferase activity compared to that in the absence of Smad overexpression was presented. The data are the mean \pm standard deviation of 5 experiments. * $p < 0.01$.

Smad2 and/or Smad4 without TGF- β 1 treatment (Fig. 4). Compared to control, luciferase activity was significantly increased in HEK293 cells by overexpression of Smad2, Smad4 or both (4.5, 5.8 and 11 fold, respectively) ($p < 0.01$). These results imply that TIM-3 promoter region may respond to TGF- β 1 stimulation through Smad2 and Smad4 involvement.

DISCUSSION

In this study, we revealed that TIM-3 mRNA expression in a human mast cell line was increased by TGF- β 1 stimulation but not by other stimuli such as interferon α and λ , TNF- α , and IL-10. TGF- β 1 affects mast cell survival and functions. TGF- β 1 inhibits IL-3-dependent mast cell proliferation and counterbalances the effect of IL-4 on mast cell survival, migration, and Fc ϵ RI expression (16,17). Also TGF- β 1 can elicit mouse mast cell protease-1 expression (18) and mast cell tryptase expression in experimental emphysema model (19). Furthermore, TGF- β 1 in both soluble and regulatory T cell-surface bound forms can escalate IL-6 production by mast cells (20). Our results (21) and the previous report by Wiener et al. (12) add another one to the effects of TGF- β 1 on mast cells i.e. induction of TIM-3 expression. Except the report by Nakae et al. (7) that TIM-3 cross-linking by an anti-TIM-3 polyclonal Ab can promote IL-4, IL-6 and IL-13 production but

suppress mast cell apoptosis, little information is currently available regarding the role of TIM-3 in mast cell function.

We also demonstrated that TIM-3 promoter $-349 \sim +144$ bp region relative to the transcription start site was crucial for the basal and TGF- β 1-induced TIM-3 transcription in HMC-1 cells. Since T3U(0.5)-luc driven luciferase activity in HMC-1 cells was greater than that driven by T3U(1.8)-luc or T3U(1.0)-luc, the DNA fragment of $-349 \sim +144$ bp seems to contain elements for basal transcription of TIM-3. Compatible to our results, Zhang et al. (22) reported that the basal TIM-3 promoter activity is localized to the region spanning -241 to $+63$ bp in YT cells, a T/NK cell line. Apparently incompatible with our previous observation that TIM-3 promoter activity driven by the $-1,362$ to $+144$ bp region was not enhanced by TGF- β 1 stimulation (21), luciferase activities under TIM-3 promoter $-349 \sim +144$ bp region and $-1,677 \sim +144$ was significantly increased in HMC-1 cells by TGF- β 1 stimulation (Fig. 3). Interestingly, the luciferase activity under TIM-3 promoter $-872 \sim +144$ was not significantly elevated by TGF- β 1 stimulation in the present study. Further study is required to investigate whether TIM-3 promoter $-1,362$ to -350 bp region may contain the element hindering the TGF- β 1 responsiveness of TIM-3 promoter $-349 \sim +144$ bp region.

We showed that overexpression of Smad2 and Smad4 up-regulated TIM-3 promoter activity in HEK293 cells. Smad2 and Smad4 are downstream signaling molecules and transcription factors of TGF- β 1 signaling (13,14). Although there are three predicted Smad binding elements in TIM-3 promoter $-349 \sim +144$ bp region, further study should be followed to clarify whether Smad2 and Smad4 are directly bound to TIM-3 promoter or indirectly involved in TIM-3 transcription in TGF- β 1 stimulated mast cells. In T cells, it was reported that two transcription factors regulated TIM-3 transcription in different modes. T-box transcription factor T-bet increases Tim-3 transcription via its interaction with Tim-3 promoter of mouse TH1 cells at approximately 400 bp upstream of the first ATG, whereas signal transducer and activator of transcription (STAT)-4 dose not bind to the Tim-3 promoter but reduces Tim-3 expression in murine T cells when it is knocked-out (23). At present, critical role of MEK, another downstream signaling molecule of TGF- β 1 signal pathway, in TIM-3 induction is revealed in HMC-1 cells stimulated with TGF- β 1 (21).

Conclusively, our results localize TIM-3 promoter activity in resting and TGF- β 1 stimulated HMC-1 cells to the region from -349 to $+144$ bp relative to the transcription start site and propose a possible regulatory role of Smad2 and Smad4

in TIM-3 transcription of mast cells.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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