Identification of Smad-dependent and -independent signaling with transforming growth factor- β type 1/2 receptor inhibition in palatogenesis

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ARTICLE INFO

Keywords:

Palatal fusion

Cleft palate

TGF- β signaling

Smad-dependent/-independent

Medial edge epithelial cell

ABSTRACT

TGF- β signaling is one of important function during palatal fusion. Three types of TGF- β receptor (T β R1, T β R2, and T β R3) have been identified, and play essential roles in mechanisms leading to palatal fusion. However, the balance between Smad-dependent/-independent signaling during palatal fusion with inhibited T β R1/2 functions is not fully understood. The objective of this study was to investigate palatal fusion via TGF- β signaling when T β R1 and T β R2, but not T β R3, were inhibited. In addition, the present study examined the functional balance between Smad-dependent/-independent signaling and related gene expression. Palatal organ cultures were treated with T β R1/2 inhibitor in vitro. Control palates were cultured without inhibitor. We observed histological phenotype of palatal fusion, and evaluation of expression pattern by Western blot or real time RT-PCR. Palatal organ cultures treated with the inhibitor did not fuse and the medial edge epithelium remained at embryonic 13 day +72 h in culture. The inhibitor decreased T β R1 and T β R2 expression by approximately 90%, but did not affect T β R3 expression. The expression of p-Smad2 and p-Smad3 was significantly decreased in treated palates compared with controls. The expression of p-Smad4 was slightly decreased in treated palates compared with controls. Smad-independent signaling was also affected by the inhibitor; p-ERK, p-JNK, and p-p38 expressions was significantly reduced in treated palates compared with controls. The expression of transcription factors (Runx1 and Msx1) and extracellular matrix proteins (MMP2/13) was also significantly decreased by inhibitor exposure. Treatment with T β R1/2 inhibitor altered the patterns of the Smad-dependent and -independent signaling pathways during palatal fusion.

1. Introduction

Transforming growth factor (TGF)- β signaling has an important function during palatogenesis.¹ TGF- β has three isoforms: TGF- β 1, - β 2, and - β 3.² Before palatal fusion, TGF- β 1 is expressed in medial edge epithelial (MEE) cells and palatal shelf mesenchymal cells.³ TGF- β 2 is also expressed in MEE and palatal mesenchymal cells in the fused stage.⁴ Various craniofacial defects, including cleft palate, have been observed in TGF- β 2 knockout mice.⁴ TGF- β 3 is strongly expressed in MEE cells.⁵ TGF- β 3-null mutant mice have completely cleft palates, despite the adequate size of the two palatal shelves.^{6,7} TGF- β 3 signaling controls the cellular differentiation required for medial edge epithelium disintegration during days 14–17 in murine embryos.⁸

TGF- β receptor isoforms include two serine/threonine receptor kinases (T β R1 and T β R2) and betaglycan (T β R3).^{2,9} Mutations in T β R1 and T β R2 lead to distinct forms of Loays-Dietz syndrome.¹⁰ During palatal fusion, T β R3 expression occurs only in MEE cells; in contrast, all palatal epithelial cells show T β R1 expression, consistent with T β R2 and TGF- β 3 expression patterns.¹¹ In addition, we showed in a previous study that palatal fusion involves the expression of all T β Rs.^{12,13}

However, the balance between Smad-dependent and -independent signaling, including the activity of related transcription factors and extracellular matrix (ECM) in palate genesis with double inhibited T β R1 and T β R2 function is not fully understood. Thus, the experimental inhibition of palatal development could exclude the signaling of all three ligands via T β R1 and T β R2 during fusion. The aim of the present

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<https://doi.org/10.1016/j.jobcr.2020.01.002>

Received 5 March 2019; Received in revised form 28 November 2019; Accepted 13 January 2020

Available online 16 January 2020

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Table 1
PCR primer sequence.

	Right	Left	Size
TβR1	CAG AGG GCA CCA CCT TAA AA	AAT GGT CCT GGC AAT TGT TC	101 bp
TβR2	TCG CTC ATC TCC ACA GTG AC	AGG CAA CAG GTC AAG TCG TT	112 bp
TβR3	ATG GTC CCC TGT GTA GCT TG	GCG GAG TAT CAG GAG TCA GC	99 bp
MMP2	GCC GCC TTT AAC TGG AGC AA	TCC CAG GCA TCT GCG ATG AG	98 bp
MMP13	GTC TTC CCC GTG TCC AAA AGA	TGA CCT GGG ATT TCC AAA AGA	105 bp
Runx1	GCG TTT GAA AGC AGG ATC TC	TAA GTC CAG CCG TTT TTG CT	120 bp
Msx1	AGC TCT GCT GCC CTA TAC CA	CAG AAG GGG TCA GAT GAG GA	102 bp
GAPDH	CAATGACCCC TTCATTGACC	GACAAGCTTCCCGTTCTCAG	106 bp

A: Western blot

B: PCR

Fig. 1. TβR1, TβR2, and TβR3 expression by treatment with TβR1/2 inhibitor. A. Western blots. B. Real time RT-PCR results. ($n = 5$, $*p < 0.05$).

study was to precisely characterize the spatial and temporal balance of Smad-dependent and -independent pathways, including ECM and transcription factor expression, by examining the functional role of TGF-βs under both of TβR1 and TβR2 inhibition in palatal MEE cells.

2. Materials and methods

2.1. Palatal organ culture

This study was approved by Nihon University School of Dentistry Animal Care and Use Committee (AP10D013 and AP17D018). Timed-pregnant Swiss-Webster mice (Slc ICR mice; Sankyo Laboratory, Tokyo,

Japan) were used for the experiments. The presence of a vaginal plug was used to determine embryonic day 0 (E0). On E13, the palatal shelves (which were positioned horizontally above the tongue, but not yet adhering to each other) were dissected from fetal murine heads under sterile conditions and placed in pairs on Millipore filters (Merck Millipore and Sigma-Aldrich, St. Louis, Missouri, USA) in correct anteroposterior orientation and with the medial edges in contact. The palatal shelves were placed in Grobstein organ culture dishes and cultured at the air–fluid interface (37 °C, 8% CO₂) in BGjib medium (GIBCO, Grand Island, NY, USA). Cultures were analyzed at 24 h (equivalent to E14 [elevated stage]) and 72 h (equivalent to E15 [fused stage]), which were found in an *in vivo* study to be critical stages in palatogenesis.¹²

Each experimental groups of palatal shelves were treated with 10, 25, and 50 nM TβR1/2 inhibitor (LY2109761; Selleck, Huston, TX, USA) at the beginning of organ culture. Control palates were not treated with inhibitor.

2.2. Histological evaluation

Frozen sections (10 μm thick) were prepared from the cultured palatal shelves at E13 + 72 h. After sectioning and slide mounting, the specimens were stained with hematoxylin and eosin (Fuji Film Wako Pure Chemical Co., Osaka, Japan).¹² Based on the results of histological examination, an inhibitor concentration of 50 nM was used for subsequent experiments to identify the effect of inhibition.

2.3. Western blotting

Palatal organ cultures were harvested for Western blot analysis at E13 + 24 h. Western blots were performed according to the procedure described previously.¹² The specific polyclonal antibodies for TβRs used were anti-TβR1, anti-TβR2, and anti-TβR3 (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Smad-dependent expression was analyzed using anti-Smad2, anti-p-Smad2, anti-Smad3, anti-p-Smad3, anti-Smad4 (1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), and anti-p-Smad4 (1:500; Invitrogen, Carlsbad, CA, USA). The

Fig. 2. Phenotypes of palatal development with TβR1/2 inhibition in culture on E13 + 72 h. A. Completely fused control palate. B. Cleft inhibitor-treated palate.

Fig. 3. Effects of T β R1/2 inhibition on Smad-dependent phosphorylation in MEE cells ($n = 5$, $*p < 0.05$). A–C. Smad2, Smad3, and Smad4 expression, respectively.

expression of non-Smad-related regulatory factors was assessed using anti-extracellular signal-regulated kinase (ERK) 1/2, anti-p-ERK1/2, anti-c-Jun N-terminal kinase (JNK), anti-p-JNK, anti-p38 mitogen-activated protein kinase (p38), and anti-p-p38 (1:250; Cell Signaling Technology, Inc.). The standard housekeeping protein GAPDH was used for control with anti-GAPDH (1:1000; Chemicon International, Temecula, CA, USA), and the ratio of GAPDH intensity was calculated for T β Rs expression.¹²

2.4. Quantitative evaluation of mRNA expression for TGF- β signaling in MEE cells

Total mRNA samples from MEE cells at E13 + 24 h were prepared and reverse transcribed into cDNA. Real-time RT-PCR was performed as

Fig. 4. Effects of T β R1/2 inhibition on Smad-independent phosphorylation in MEE cells ($n = 5$, $*p < 0.05$). A–C. ERK1/2, JNK, and p38 expression, respectively.

described previously¹² to investigate the mRNA expression of T β R1, T β R2, and T β R3. In addition, to identify TGF- β downstream signaling following T β R1/2 inhibitor treatment during palatal fusion, the expression levels of Runx-related transcription factor (Runx) 1 and Msh homeobox (Msx) 1 were determined by real-time RT-PCR analysis. To identify the extracellular matrix expressions related with TGF- β signaling, the expression levels of matrix metalloproteinase (MMP) 2 and MMP13 were also determined by real-time RT-PCR analysis. The primer pairs and product sizes are listed in Table 1. The levels of mRNA expression were calculated and normalized to the level of GAPDH mRNA.¹²

2.5. Statistical analysis

Results from multiple groups were compared with analysis of

Fig. 5. Effects of T β R1/2 inhibition on the expression of transcription factors and ECM in MEE cells ($n = 5$, $*p < 0.05$). A–D. Runx1, Msx1, MMP2, and MMP13 expression, respectively.

variance and Tukey's honestly significant difference tests. The level of significance was set at $p < 0.05$. Western blot and PCR data were analyzed with SPSS software (IBM Corporation, Armonk, NY, USA) to compare target gene expression in control and treated palates.¹²

3. Results

3.1. Effect of T β R1/2 inhibitor

Treatment with 50 nM T β R1/2 inhibitor reduced the protein expression of T β R1 and T β R2 by approximately 90% compared with the control (Fig. 1A). GAPDH was not affected in the experimental or control treatment groups. In addition, no significant difference in the protein expression of T β R3 was observed between control and treated palates (Fig. 1C).

The inhibitor had a dose-dependent effect, with progressively larger reductions in the mRNA expression of T β R1 and T β R2 occurring with 10-, 25-, and 50-nM treatments (Fig. 1B). In contrast, T β R3 mRNA expression was detected at the same level in control and treated palates, and was not influenced by the inhibitor (Fig. 1A and B).

3.2. Palatal fusion phenotype following treatment with T β R1/2 inhibitor

Histological examination revealed complete fusion of the palatal shelves at E13 + 72 h in the control group (Fig. 2A). In contrast, palatal shelves in the treatment group were not fused at the same time point (Fig. 2B). This phenotype was similar to that of palates of TGF- β 3 knockout null mice.^{6,7}

3.3. Effect of inhibited T β R1/2 function on Smad-dependent phosphorylation in MEE cells

Following treatment with T β R1/2 inhibitor, the relative amounts of

p-Smad2/Smad2 were decreased significantly compared with the control ($p < 0.05$; Fig. 3A). The relative amount of p-Smad3/Smad3 and p-Smad4/Smad4 expression was slightly lesser than in the control, but a significant difference was observed only between the control and 50-nM treatment groups ($p < 0.05$; Fig. 3B and C). The inhibitor had a dose-dependent effect, with progressively larger reductions in the phosphorylation of Smad2/3/4 occurring with 10-, 25-, and 50-nM treatments (Fig. 3A, B, C).

3.4. Effect of inhibited T β R1/2 function on Smad-independent (ERK/p38/TAK1/JNK) phosphorylation in MEE cells

Following treatment with T β R1/2 inhibitor, the relative amounts of p-ERK/ERK, p-JNK/JNK, and p-p38/p38 were decreased significantly compared with the control ($p < 0.05$; Fig. 4A–C). The inhibitor had a dose-dependent effect, with progressively increase reductions in the mRNA phosphorylation of ERK/JNK/p38 occurring with 10-, 25-, and 50-nM treatments (Fig. 4B).

3.5. Expression of transcription factors (Runx1 and Msx1) and ECM proteins (MMP2 and MMP13)

Following treatment with T β R1/2 inhibitor, the relative amounts of the transcription factors Runx1 and Msx1 were decreased significantly compared with the control ($p < 0.05$; Fig. 5A and B). MMP2 and MMP13 expression was also decreased significantly by T β R1/2 inhibitor treatment compared with the control ($p < 0.05$; Fig. 5C and D).

4. Discussion

TGF- β cytokines induce cellular action by binding to specific receptors on cell surfaces. Our previous study, siRNA-induced reduction of either T β R1 or T β R2 expression produced partial clefting in the

Fig. 6. Diagrammatically show TGF- β signalling cascade events related with Smad-dependent and -independent with their interactions.

central region of the palate,¹³ and reduction of T β R3 expression delayed palatal fusion compared with the control.^{12,13}

Previous studies have shown that the activation and stimulation of T β R3 facilitates the binding of T β R2, which subsequently recruits and activates the phosphorylation of T β R1.¹⁴ The observation in the present study that T β R1/2 inhibitor affected the downstream signaling pathway of TGF- β 3, particularly Smad2, was consistent with previous findings, and further identifies a role for Smad2 phosphorylation in palatogenesis.^{15,16} In a previous study, TGF- β 3 knockout mutant palates showed significantly decreased phosphorylation of Smad2, but unchanged Smad3 expression.¹⁵

Our new results revealed that T β R1/2 inhibition using LY2109761 reduced phospho-Smad3 levels during palatal fusion, with no change in the total amounts of Smad3. These results suggest that TGF- β 1 or TGF- β 2 influences Smad3 phosphorylation during palatal fusion. In addition, we found that the phosphorylation of Smad4 was decreased slightly in treated palates compared with the control. Therefore, Smad4 phosphorylation as co-Smad could influence palatal fusion, and it may be rescued by other signaling pathways, such as those of bone morphogenetic proteins (BMPs) and interferon regulatory factors, even when TGF- β signaling is deleted.^{17,18}

T β Rs activate signaling pathways that regulate Smad-dependent signaling and induce Smad-independent TGF- β responses.¹⁹ Regarding Smad-independent signaling during palatal fusion, the relative amounts of p-ERK/p-p38/p-JNK were significantly and dose-dependently decreased in treated palates compared with controls in this study. These results could be clarified p-ERK/p-p38/p-JNK molecules are activated in Smad-independent signaling during palatal fusion (Fig. 6).

Runx1 is expressed in MEE cells during palatal development, and Runx1 null mutant mice have cleft palates.²⁰ Runx1 expression in palatal epithelium can be induced by fibroblast growth factor (FGF) 18, demonstrating the role of mesenchymal FGF18 signaling in this process.^{20,21} In this study, the inhibitor treatment significantly reduced the expression of Runx1, indicating that Runx1 and Smad-dependent signaling are both involved in palatogenesis via the interaction of TGF- β and FGF signaling.^{20,21} Msx1 expression is also associated strongly with palatal fusion; The deletion of Msx1 in null-mutant was significantly reduced both BMP2 and BMP4 expressions.²² Our study identified that Msx1 expression was significantly reduced by T β R1/2 inhibition, suggesting an association with cross-talk and/or interaction between TGF- β and BMP signaling.

During palatal fusion, the expression of ECM proteins, such as MMPs

and tissue inhibitors or metalloproteinases (TIMPs), was induced in palatal MEE cells, and MMP13 is responsive to TGF- β 3 stimulation around the midline seam.^{23–25} The ECM proteins as MMP2 and MMP13 play roles in cell migration, proliferation, and differentiation.²⁴ Thus, our finding results identified that TGF- β and ECM signaling are linked and could be associated with epithelial–mesenchymal transition during palatal development.

In conclusion, the observed reduction of T β R1/2 and working T β R3 function suggests that T β R1 and T β R2 are important modulators of TGF- β function, that T β R3 supports T β R1/2 function, and that this signaling pathway is associated strongly with the palatal fusion phenotype. In addition, we showed in Fig. 6 that the balance of Smad-dependent and -independent signaling is also important in palatogenesis. Given the close correlation of MEE TGF- β and T β R1/2 expression patterns during palatal development, T β R3 appears to play a crucial role in fusion-inducing mechanisms. Our results demonstrate that T β R1/2 inhibition can affect downstream TGF- β signaling, including the Smad-dependent and -independent phosphorylation pathways, during palatal development. The T β R1/2 complex plays a role in normal palatal fusion that can be circumvented under conditions with high levels of TGF- β , which supports a role of T β R1/2/3 in increasing the local concentrations of TGF- β in the region of the medial edge epithelium.

Funding

This work was supported by a MEXT grant for multidisciplinary research projects and a MEXT Grant-in-Aid for Scientific Research (C) (nos. 23593046 and 26463100).

Grant information

MEXT grants for multidisciplinary research projects and Grant-in-Aid for Scientific Research (C) 26463100.

Ethical approval

This work was approved by the Ethical Committee of Nihon University.

Declaration of competing interest

No conflict.

Acknowledgments

The authors thank all of the study subjects and research staff who participated in this work.

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