Excessive retinoic acid inhibit mouse embryonic palate mesenchymal cell growth through involvement of Smad signaling

Huanhuan Zhang, Xiaozhuan Liu, Zhan Gao, Zhitao Li, Zengli Yu, Jun Yin, Yuchang Tao and Lingling Cui

ABSTRACT

All-trans retinoic acid (atRA), the oxidative metabolite of retinoic acid (RA), is essential for palatogenesis. Overdose RA is capable of inducing cleft palate in mice and humans. Normal embryonic palatal mesenchymal (EPM) cell growth is crucial for shelf growth. Smad signaling is involved in many biological processes. However, it is not much clear if atRA could affect Smad signaling during EPM cells growth. In this study, the timed pregnant mice with maternal administration of 100 mg/kg body weight of RA by gastric intubation were cervical dislocation executed to evaluate growth changes of palatal shelves by hematoxylin and eosin (H&E) staining. At the same time, a primary mouse EPM (MEPM) cell culture model was also established. MEPM cells were treated with atRA (0.1, 0.5, 1, 5 and 10 μM) for 24, 48 and 72 h. The results indicated that the sizes of the shelves were smaller than those in control. AtRA inhibited MEPM cell growth with both increasing concentration and increasing incubation time, especially at 72 h in vitro. Moreover, atRA significantly increased the mRNA and protein expression levels of Smad7 (P < .05), but the mRNA and protein expression levels of PCNA were reduced (P < .05). We also found atRA inhibited phosphorylation of Smad2 compared with untreated group (P < .05). However, the protein and mRNA levels of Smad2 did not change both in atRA-treated and untreated group (P > .05). We demonstrated that RA induced inhibition of MEPM cell growth that could cause cleft palate partly by down-regulation of Smad pathway.

ARTICLE HISTORY

Received 23 November 2015
Revised 19 February 2016
Accepted 23 February 2016

KEYWORDS

Retinoic acid; MEPM; cell growth; Smad

Introduction

In mammals, the palatal shelf is an important structure and plays an important role in the essential functions of breathing and feeding. Palatoquadrate cartilage formation is initiated with condensation of mesenchymal cells during embryonic development. The secondary palate develops from bilateral outgrowth of the maxillary processes, it increases in size also mainly depends on condensation of mesenchymal cells which results in palatal growth, elevation and fusion. It is no doubt disruption of mesenchymal cell growth will lead to cleft palate. Retinoic acid (RA) is a synthetic retinoid used for the treatment of psoriasis. Overdose RA could cause cleft palate both in mice and humans. In vivo, we found RA induced growth retardation of the palatal shelves from gestation day 13 (Figure 1). All-trans retinoic acid (atRA) is an oxidative of RA. Previous studies demonstrated that atRA could induce mouse embryonic palatal mesenchymal (MEPM) cell cycle arrest (Yu et al. 2005). Both atRA and Smad protein are implicated in a wide range of cellular functions in craniofacial development (Opperman et al. 1997; Hu et al. 2013). TGF-βs existed in three isoforms: TGF-β1, TGF-β2 and TGF-β3, and were encoded by three different genes (Massagué 2000). TGF-β/Smad pathway played a crucial role in the regulation of cell proliferation and growth (Zhu et al. 2012; Parada et al. 2013). Smad pathway was downstream of TGF-β receptors. TGF-βs bound to the TGF-β receptor type II (TGF-β RII) and activated TGF-β receptor type I (TGF-β RI), then subsequently triggered the phosphorylation of Smad2/3. The phosphorylated Smad2/3 would bind and activate co-Smad, Smad4, then translocate into the nucleus to regulate the TGF-β-responsive genes (Nakao et al. 1997). Smad7 acted as an inhibitor of Smad signaling.

Previous studies showed that Smad signaling played an important role in the regulation of growth of normal and cancer cells (Ackermans et al. 2011; Tian et al. 2011). However, it is not clear whether Smad signaling is involved in the process of atRA inhibiting the growth of MEPM cells. We, therefore, hypothesized that atRA induced inhibition of MEPM cell growth that could cause cleft palate at least in part by disturbing Smad pathway.
Materials and methods
Animals
C57BL/6J mice were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). This study was performed in Zhengzhou University. Our experiments were permitted by the Medical Ethical Committee of Zhengzhou University, China, and all the procedures were conducted in accordance with the ethical standards. Female mice were crossed with fertile males overnight. Observation of a vaginal plug was designated as gestation day 0. All pregnant females at gestation day 10 were randomly divided into two groups for treatment: RA (Sigma®, United States) was dissolved in corn oil by intragastric administration at 100 mg/kg, and 10 ml/kg corn oil only used as control.

Histological analysis
Timed pregnant mice were cervical dislocation executed on gestation day 13, gestation day 14, and gestation day 15. All embryonic heads were fixed in 4% paraformaldehyde (PFA) and processed into paraffin-embedded serial sections by using routine procedures. To evaluate growth changes of palatal shelves, deparaffinized sections were stained with hematoxylin and eosin (H&E) using standard procedures.

Cell culture and treatment
Primary MEPM cell culture: the primary method used for MEPM cell culture from gestation day 13 mouse embryos was introduced in detail (Yu et al. 2005). Cells were then cultured in flasks in DMEM/F12 medium (Hylen®, Logan, UT, United States) containing 10% fetal calf serum (FBS, Hylen®, Logan, UT, United States) at 37°C in a 95% air/5% CO2 atmosphere with media replaced every other day and then subcultured at the exponential growth stage (80% confluence).

MTT assays
The third generation cells (5 × 10^3 cells per well) were seeded in 96-well microtiter plates (Nunc®, Denmark), and treated with atRA (0.1–10 μM) (Sigma®, United States) and vehicle (control group). After different treatments for 24, 48, and 72 h, the plates were incubated for additional 4 h at 37°C; 20 μl MTT (Genview®, CA, United States) was added to each well, and then MTT solution was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 150 μl DMSO was added to each well. The absorbance was read at 490 nm on a Biotek automatic micro well plate reader, DMSO as the blank.

Semi-quantitative PCR analysis
The mRNA expression levels of TGF-β/Smad pathway-related specific molecules were determined by semi-quantitative RT-PCR analysis. The total RNA was isolated from MEPM cells by using Trizol (Life Technologies®, United States) according to the manufacturer’s protocol. Briefly, 50 mg palatal tissues were preserved in 1 ml Trizol. Then the tissues were fully cracked by using the homogenizer. The concentration of extracted RNA was tested by photometric measurement (Thermo Scientific®, United States). The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific®, United States) was used for cDNA synthesis; 1 μg of isolated RNA was used as a template to generate first strand cDNA according to the manufacturer’s protocol. The reaction condition was 25°C for 5 min, followed by 60 min at 42°C, and terminated the reaction by heating at 70°C for 5 min. The master mix for PCR reaction contained: 1 μg of cDNA, 10 μM primers and 25 U 2 × Taq MasterMix (Cwbiotech®, Beijing, China). The mixture was heated at 94°C for

Figure 1. Coronal sections were prepared on gestation day 13, gestation day 14, and gestation day 15. All control palatal shelves elevated to a horizontal position above dorsum of the tongue (a), and completely contacted, adhered and fused along midline (c), then formed a complete palate eventually (e). But the palatal shelves elevation were delayed on gestation day 13 in RA-treated group (b), and the size of the shelves were small (d), a large gap presented as a wider cleft phenotype with RA treatment on gestation day 15 (f). ps, palatal shelf; T, tongue; N, nose. Scale bars represent 400 μm (A–F).
2 min and 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and then a single 2 min extension at 72 °C. PCR amplification was carried out on the LabCycler Basic Plus (SENSO®, German) with gene-specific primers. Then the PCR amplification products were analyzed using agarose gel electrophoresis. Specific primers for Smad2, Smad7, PCNA and β-actin were used and the corresponding primers were listed (Table 1). β-actin was used as an experimental control. The analysis was repeated three times independently.

Western blot analysis

All lysates were prepared from MEPM cells by using 2 × SDS-lysis buffer, and supplemented with protease inhibitors (M250, Amresco, Ohio, United States) and phosphates inhibitors (WB0117, Weiao Biotech®, Shanghai, China). Protein content was determined by using a standard BCA protein assay kit (Dingguo®, Beijing, China); 50 μg proteins were fractionated on 12% SDS-PAGE, electroblotted to nitrocellulose membranes. After blocking with 5% nonfat milk, the membranes were immunoblotted with specific primary antibodies: Smad2 (ab71109, Abcam*, MA, United States), p-Smad2 (sc101801, Santa Cruz*, CA, United States), Smad7 (sc-365846, Santa Cruz*, CA, United States), PCNA (Dingguo®, Beijing, China) and β-actin (WD03056, Weiao Biotech®, Shanghai, China). β-actin was probed as a loading control. Then washing and incubation with HRP-conjugated secondary antibodies (sc-2004 or sc-2005, Santa Cruz*, CA, United States), membrane-binding HRP was detected by enhanced chemiluminescence (ECL Kit, Dingguo®, Beijing, China) and then exposed to Kodak film. The intensity of the bands was analyzed by densitometry.

Statistical analysis

Data from at least three replicates for each parameter were evaluated and analyzed for significance by SPSS 13.0. All values in each group were expressed as the mean ± SD. Statistical analysis between groups was performed using one-way ANOVA. Statistical analysis for the effect of atRA treatment on growth of MEPM cells was performed using repeated measure ANOVA. P < .05 was considered statistically significant. The data shown in the figures were the relative fold changes.

Results

Histology of palatal shelves

As shown in Figure 1, the palatal shelves in controlbilaterally elevated and the open space of the oral-nasal was larger compared with experimental group on gestation day 13. All control shelves elevated to a horizontal position above the dorsum of the tongue, and completely contacted, adhered and fused along their midline on gestation day 14, whereas the palatal shelves of RA-treated embryos were still not elevated and the sizes of the shelves were smaller than palatal shelves in controls. Compared with the complete secondary palate formation in controls, a large gap eventually presented as a wider cleft phenotype in RA-treated group on gestation day 15.

The effect of atRA treatment on growth of MEPM cells

In order to investigate the effect of atRA on the growth of MEPM cells, cells were exposed to the vehicle or indicated concentrations (0.1, 0.5, 1, 5 and 10 μM) of atRA for 24, 48 and 72 h, and the quantification of MEPM cell growth and viability were tested by MTT assay. As shown in Figure 2, viability of MEPM cells treated with atRA at 24 and 48 h were decreased with increasing concentration. But atRA distinctly inhibited cell growth in a dose-dependent manner at 72 h.

Altered mRNA expression of Smad pathway specific molecules by atRA

Smad7 and Smad2 were TGF-β/Smad pathway downstream signal molecules. Thus, in this set of experiments, we explored the effects of atRA on mRNA expression level of Smad7 and Smad2. Moreover, PCNA were also tested. All primers were listed (Table 1). As shown in Figure 3(a) and 3(b), atRA (5 μM) significantly increased the mRNA level of Smad7, decreased mRNA level of PCNA. But the levels of Smad2 were unchanged in both groups.

Down-regulation of Smad pathway by atRA

Since TGF-β signaling was mainly mediated by Smad proteins, we doubted the expression of Smad proteins may be also altered in the process of atRA inhibiting MEPM

Table 1. List of primers used in this study

| Primer Name | Primer Sequence (5’-3’) | F=Forward, R=Reverse |
|-------------|-------------------------|---------------------|
| Smad2       | F=5’-CGGAGATTCTAACAAGAACCTG-3’ | R=5’-TGCTTGACATCGCATGAA-3’ |
| Smad7       | F=5’TGTGCTGTGAATCTCGGGAAGA-3’ | R=5’-TGCAAGTGGAAGACTGGAAC-3’ |
| PCNA        | F=5’-TCAGTCACTCATCGAAAGA-3’ | R=5’-TGCAAGTGGAAGACTGGAAC-3’ |
| β-actin     | F=5’-ATCATGTGGACCTCACAAC-3’ | R=5’-CATCTCTGCTGGACTCA-3’ |
cell growth. We investigated the protein expression levels both in atRA-treated and untreated group. As shown in Figure 4(a) and 4(b), atRA decreased p-Smad2 and PCNA, but increased Smad7 protein level. The protein levels of Smad2 remain unchanged in two groups.

**Discussion**

During embryonic development, mesenchymal cell proliferation was the main process in shelf outgrowth. Disruption of this process led to cleft palate. Both deficiency and overdose of RA could cause cleft palate in mice and humans (Ackermans et al. 2011). Embryos exposed to RA showed 100% incidence of the cleft palate. At the time of palatal shelf outgrowth, overdose of RA resulted in growth retardation of bilateral palate shelves in mice (Yoshikawa et al. 1987). In this study, we found that atRA permanently impeded palatal shelves growth in vivo on gestation day 13 (Figure 1(b)), and the size of palate shelves treated with RA were smaller when compared with control group. The palatal shelves of RA-treated embryos on gestation day 14 were not elevated (Figure 1(d)). In previous study, excessive exogenous atRA could adversely affect chondrogenesis in vitro (Yu & Xing 2006) and inhibit palate growth which caused cleft palate (Yoshikawa et al. 1987). We showed that overdose RA caused growth retardation and growth retardation of the palatal shelves appeared played a major role in RA-induced cleft palate. This was also the main cause for incidence of cleft palate (Choi et al. 2011). In our study, we also observed atRA could significantly inhibit MEPM cell growth especially in a dose- and time-dependent manner at 72 h in vitro (Figure 2). Concentrations of atRA in some studies were excessively high (up to 100 μM) (Suwa et al. 2001), some researchers used atRA at 5 μM and 10 μM to mimic the teratogenic concentration (Yu et al. 2005). Seewaldt demonstrated that 10 μM atRA was cytotoxic (Seewaldt et al. 1997). According to our data, we treated with atRA at 5 μM for next assays. Further semi-quantitative PCR analysis and western blot analysis revealed that the mRNA and protein expression of proliferating cell nuclear antigen (PCNA) were decreased by 5 μM atRA at 72 h (Figures 3 and 4). All these data implied that overdose of atRA could inhibit MEPM cell growth which could cause cleft palate.

To date, much effort had been spent to study the mechanism of atRA inhibiting MEPM cell proliferation (Hu et al. 2013). However, the exact mechanism of atRA inhibited MEPM cell growth was not fully understood. TGF-β/Smad signaling stimulated proliferation and inhibited terminal differentiation of chondrocytes during chondrogenesis. Smad pathway was downstream of TGF-β receptors, and RA could affect the expression of TGF-βs (Abbott & Birnbaum 1990). TGF-β initiated its cellular actions by binding to and activating TGFβRII and was mediated by Smad transcription factors. Zhan et al. blocked TGF-β/Smad signaling could inhibit the proliferation and phenotypic transformation of rat cardiac fibroblasts (Zhan et al. 2014). TGF-β/Smad pathway played a critical role in RA-elicited cellular response (Li et al. 2014). Smad pathway was antagonized by Smad7, which interacted with TGFβRI to inhibit the phosphorylation of Smad2/3. Because Smad7 acted as an inhibitor of Smad signaling and Smad7 overexpression led to pancreatic endocrine hypoplasia (Smart et al. 2006), we examined the present level of Smad7 in the MEPM cells after treated with 5 μM atRA. We found mRNA and protein expression levels of Smad7 were both increased (Figures 3 and 4). Our results showed atRA may up-regulate Smad7 to inhibit MEPM cell growth. Smad7 and Smad2 both were TGF-β/Smad pathway downstream signal molecules. Smad2 was well-known key mediator of the TGF-β/Smad pathway and expressed in epithelium and mesenchyme. Smad2 and Smad3 were structurally very closely related and both were involved in signaling from TGF-β, Smad3 could form a complex together with Smad2 in order to regulate the target genes of TGF-βs. Over-expression of Smad2 inhibited the proliferation of junctional epithelium cells by down-regulating c-Myc and up-regulating p15 and p27 which leading to cell-cycle arrest (Alotaibi et al. 2014), and inhibited oral
epithelial cell proliferation by increasing p15 and p21 (Shimoe et al. 2014). However, Smad2 was not required for TGF-β-stimulated growth inhibition in hepatocytes (Ju et al. 2006). Our results showed that Smad2 did not change in MEPM cells treated with or without atRA (Figure 3 and 4). MiR-200b was expressed in the medial edge epithelium (MEE) and palatal mesenchyme, and played a crucial role in regulating Smad2 (Shin et al. 2012). AtRA may have no regulatory effects on miR-200b in the process of MEPM cell growth. But further studies should be performed to investigate the relationship between atRA and miR-200b in palatal development. The phospho-Smad2/3 would bind and activate co-Smad, Smad4, and then translocated into the nucleus to regulate the TGF-β-responsive genes (Nakao et al. 1997). Therefore, the level of phospho-Smad2 was also pivotal for Smad pathway. In our experiment, the expression level of phospho-Smad2 in MEPM cell was reduced. Because lack of phospho-Smad2, TGF-β/Smad pathway may not be activated so that endogenous TGF-βs cannot regulate MEPM cell growth normally. These results demonstrated that atRA produced a negative effect on Smad signaling in the process of MEPM cell growth.

In summary, this study demonstrated RA induced inhibition of MEPM cell growth that caused cleft palate in part by down-regulating Smad pathway. Moreover, we speculated that Smad2 and Smad7 may be target genes of atRA during MEPM cell growth, but further studies should be performed. Next, we will abort Smad2 and Smad7 to verify the role of TGF-β/Smad pathway in the process of MEPM cell growth.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This study was funded by National Natural Science Foundation of China Nos 21407128, 21577119 and U1304327.
References

Abbott BD, Birnbaum LS. 1990. Retinoic acid-induced alterations in the expression of growth factors in embryonic mouse palatal shelves. Teratology. 42:597–610.

Ackermans MM, Zhou H, Carels CE, Wagener FA, Von den Hoff JW. 2011. Vitamin A and clefting: putative biological mechanisms. Nutr Rev. 69:613–624.

Alotaibi MK, Kitase Y, Shuler CF. 2014. Smad2 overexpression reduces the proliferation of the junctional epithelium. J Dent Res. 93:898–903.

Choi JW, Park HW, Kwon YJ, Park BY. 2011. Role of Apoptosis in Retinoic Acid-Induced Cleft Palate. J Craniofac Surg. 22:1567–1571.

Ju W, Ogawa A, Heyer J, Nierhof D, Yu L, Kucherlapati R, Shafritz DA, Böttinger EP. 2006. Deletion of Smad2 in mouse liver reveals novel functions in Hepatocyte growth and differentiation. Mol Cell Biol. 26:654–667.

Li X, Yin X, Gao Z, Zhang H, Liu X, Pan X, Li N, Yu Z. 2014. Retinoic acid remodels extracellular matrix (ECM) of cultured human fetal palatal mesenchymal cells (hFPMCs) through down-regulation of TGF-/Smad signaling. Toxicol Lett. 225:208–215.

Massagué J. 2000. How cells read TGF-beta signals. Nat Rev Mol Cell Biol. 1:169–178.

Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P. 1997. TGFβ receptor-mediated signaling through Smad3 and Smad4. EMBO J. 16:5353–5362.

Opperman LA, Nolen AA, Ogle RC. 1997. TGF-β1, TGF-β2, and TGF-β3 exhibit distinct patterns of expression during cranial suture formation and obliteration in vivo and in vitro. J Bone Miner Res. 12:301–310.

Parada C, Li J, Iwata J, Chai Y. 2013. CTGF mediates Smad-dependent transforming growth factor signaling to regulate mesenchymal cell proliferation during palate development. Mol Cell Biol. 33:3482–3493.

Seewaldt VL, Kim JH, Caldwell LE, Johnson BS, Swisshelm K, Collins SJ. 1997. All-trans-retinoic acid mediates G1 arrest but not apoptosis of normal human mammary epithelial cells. Cell Growth Differ. 8:631–641.

Shimoe M, Yamamoto T, Shiomi N, Tomikawa K, Hongo S, Yamashiro K, Yamaguchi T, Maeda H, Takashiba S. 2014. Overexpression of Smad2 inhibits proliferation of gingival epithelial cells. J Periodontal Res. 49:290–298.

Shin JO, Lee JM, Cho KW, Kwak S, Kwon HJ, Lee MJ, Cho SW, Kim KS, Jung HS. 2012. MiR-200b is involved in Tgf-β signaling to regulate mammalian palate development. Histochem Cell Biol. 137:67–78.

Smart NG, Apelqvist AA, Gu X, Harmon EB, Topper JN, MacDonald RJ, Kim SK. 2006. Conditional expression of Smad7 in pancreatic beta cells disrupts TGF-beta signaling and induces reversible diabetes mellitus. PLoS Biol. 4:e39.

Suwa F, Jin Y, Lu H, Li X, Tipoe GL, Lau TY, Tamada Y, Kuroki K, Fang YR. 2001. Alternation of apoptosis in cleft palate formation and ectomesenchymal stem cell influenced by retinoic acid. Okajimas Folia Anat Jap. 78:179–186.

Tian M, Neil JR, Schiemann WP. 2001. Transforming growth factor-β and the hallmarks of cancer. Cell Signal. 23:951–962.

Yoshikawa H, Kukita T, Kurisu K, Tashiro H. 1987. Effect of retinoic acid on in vitro proliferation activity and glycosaminoglycan synthesis of mesenchymal cells from palatal shelves of mouse fetuses. J Craniofac Genet Dev Biol. 17:45–51.

Yu Z, Lin J, Xiao Y, Han J, Zhang X, Jia H, Tang Y, Li Y. 2005. Induction of cellcycle arrest by all-trans retinoic acid in mouse embryonic palatal mesenchymal (MEPM) cells. Toxicol Sci. 83:349–354.

Yu Z, Xing Y. 2006. All-trans retinoic acid inhibited chondrogenesis of mouse embryonic Palate mesenchymal cells by down-regulation of TGF-β/Smad signaling. Biochem Biophys Res Commun. 340:929–934.

Zhou CY, Tang JH, Zhou DX, Li ZH. 2014. Effects of tanshinone IIA on the transforming growth factor β1/Smad signaling pathway in rat cardiac fibroblasts. Indian J Pharmacol. 46:633–638.