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Retinoic acid remodels extracellular matrix (ECM) of cultured human fetal palate mesenchymal cells (hFPMCs) through down-regulation of TGF-β/Smad signaling

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HIGHLIGHTS

- RA dose-dependently inhibited cell proliferation and mRNA and protein levels of ECM components.
- RA increased the ratio of MMP2/TIMP2 mRNA expression.
- RA inhibited TGF-β/Smad pathway.
- Activation of TGF-β/Smad signaling attenuated cellular responses of RA.

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ABSTRACT

The regulation of extracellular matrix (ECM) by retinoic acid (RA) is interesting in light of the fact that the ECM plays an essential role in morphogenesis and palatal shelf elevation. In the current study, we explored the effect of RA overexposure on ECM and the probable mechanisms in cultured human fetal palate mesenchymal cells (hFPMCs). RA dose-dependently inhibited cell proliferation and mRNA and protein levels of ECM components fibronectin, tenascin C and fibrillin-2. Zymography revealed that MMP-2 activity was suppressed by RA. Further analysis revealed that mRNA levels of MMP2 and TIMP2 were decreased, while the MMP2/TIMP2 mRNA ratio was increased, which might facilitate the ECM degradation. Because of the pivotal role of TGF-β/Smad pathway in palatogenesis we therefore checked the effect of RA on TGF-β/Smad signaling. The results indicated RA treatment increased Smad7 expression and decreased the levels of TGF-β1, TGF-β3, TGF-β type II receptor (TβRII) and phosphorylated Smad2 and Smad3. Activation of the Smad pathways by either exogenous TGF-β3 or recombinant adenoviruses for Smad3 attenuated RA-induced inhibition of cell proliferation and ECM components and rescued the RA-altered MMP2/TIMP2 mRNA ratio. In conclusion, these findings suggested that RA overexposure inhibited cell proliferation and disrupted the ECM network through down-regulation of TGF-β/Smad pathway.

1. Introduction

Palatal shelf elevation and fusion are pivotal events during palatogenesis. Proper shelf size is important for both of these events to occur. Proliferation is the main process in shelf outgrowth, while ECM production is crucial in palatal shelf elevation. Small shelves generally fail to elevate fully, and invariably fail to make contact at the midline for fusion to be triggered. The shelves increase in size by mesenchymal cell proliferation and by the production and hydration of extracellular matrix (ECM) components (Meng et al., 2009).

ECM molecules play a critical role in the morphogenesis of the secondary palate, which form a stable framework to which cells

Abbreviations: atRA, all-trans retinoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF-β, Transforming Growth Factor β; TβRII, TGF-β type II receptor; FN, fibronectin; TNC, tenascin C; FB2, fibrillin-2; ECM, extracellular matrix; MMP2, matrix metalloproteinase 2; TIMP2, tissue inhibitor of metalloproteinase 2.

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adhere via integrin receptors (d'Amaro et al., 2012). Fibrillar collagens and large proteoglycans are major structural entities of ECM. More minor ECM components are also essential components of the ECM. They form aggregates that bind large amounts of water, and the resulting swelling pressure is the driving force behind shelf elevation (Galloway et al., 2013). In addition, they have important regulatory functions: they promote cell adhesion (fibronectin, laminin) (Chiquet-Ehrismann and Chiquet, 2003), modulate cell sorting and motility (tenascins) (Kaartinen and Warburton, 2003; Fagman et al., 2003), control collagen matrix assembly, or store growth factors and present them to cells (fibrillins) (Zhang et al., 2005).

It is generally believed that the balance between MMPs and TIMPs is among the critical determinants that control the integrity of the ECM and subsequently affect cell fate (Li et al., 2013; Mroczko et al., 2013). Most MMPs are secreted from cells as zymogens and their activity in the extracellular matrix is controlled by TIMPs (Coronato et al., 2012). MMP-2 (gelatinase A) mainly degrades gelatins, non-fibrillar collagens IV, V, VII, fibronectin, laminin, and elastin. TIMP-2 is known to preferentially interact with MMP-2, both of which are present within the palatal shelf during the course of palatal morphogenesis in vivo and that their expression is developmentally regulated by TGF-β family (Chung et al., 2012; Blavier et al., 2001). Indeed, the impaired palatogenesis in the absence of TGFβ3 is accompanied by reduced glycoproteins abundance, which could reflect enhanced degradation mediated by increased MMP and decreased TIMP activity (Ackermans et al., 2011; d'Amaro et al., 2012).

The three isoforms of TGF-β (β1, β2 and β3) are all expressed in the palatal shelves at different stages of palatogenesis (Meng et al., 2009). TGF-β typically signal via heterotetrameric receptor complexes composed of two type I (TGF-βRI) and two type II (TGF-βRII) receptors, and their downstream mediators (known as Smads). On TGF-β stimulation, Smad2, and Smad3 undergo phosphorylation, triggering an interaction with Smad4. The Smad complex translocates into the nucleus to regulate the target genes (e.g., fibronectin) and directs cell transactivation. The actions of TGF-β are antagonized by Smad7, which interacts stably with TβRI to prevent phosphorylation and activation of receptor-regulated Smad2/3, thereby blocking TGF-β signaling (Zi et al., 2012; Studer et al., 2012).

The vitamin A metabolite, retinoic acid (RA), is an important regulator of embryogenesis. RA regulates proliferation, differentiation, and apoptosis during the morphogenesis of embryonic structures (Schilling et al., 2012). In pregnant mice, RA overexposure reduces the growth of the palatal shelves in the embryo (Zhang et al., 2003). This seems to be a consequence of reduced mesenchymal cell proliferation as well as inhibition of ECM production (Ackermans et al., 2011). However, there has been little investigation into the association of RA overexposure with the relative amount of different ECM glycoproteins, as well as the underlying molecular mechanisms. In the current study, by using culture of human fetal palatal mesenchymal cells (hFPMCs), we for the first time demonstrated that atRA treatment dose-dependently inhibited cell proliferation and expressions of ECMs fibronectin, tenascin C and fibrillin2 by modulation of MMP2 and TIMP2 through down-regulation of TGF-β/Smad signaling.

2. Materials and methods

2.1. Cell culture

Fetuses were obtained from women undergoing termination of pregnancy (aging from 7 to 8 weeks of amenorrhea). The women consented to the use of tissue for scientific research after approval by the Medical Ethical Committee of Zhengzhou University, China. Tips of elevated palatal shelves were removed as pairs from fetus and dissociated with 0.25% trypsin/0.1% EDTA in PBS for 10 min at 37 °C. Digested samples were briefly triturated, filtrated through 70-μm mesh, and cells were maintained in DMEM medium containing 5% FBS (Sigma, St. Louis, MO) in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. Experiments utilizing primary cultures were initiated at 24 h and the cells were then approximately 50% confluent. All-trans RA (atRA, Sigma, St. Louis, MO) was dissolved in DMSO to produce 0.1 mol/l of storage solution and added to the culture (the final concentration of DMSO was less than 0.02%, v/v). Control cells were maintained under the same conditions as treated cells but without the addition of atRA (the culture medium containing 0.02% DMSO). All experiments were performed in triplicates and repeated three times.

2.2. Cell proliferation

Cells were plated in 12-well plates and treated with indicated concentrations of atRA for 6 h, 12 h, 24 h, 36 h and 48 h, respectively. Cell proliferation was detected by MTT assay as instructed by the manufacturer. Briefly, after incubation with MTT (0.5 mg/ml) for 4 h at 37 °C, the medium was discarded, wells washed with PBS and formazan crystals dissolved in DMSO. The absorbance was measured using SpectraMax 190 (Molecular Devices, CA, USA) at 520 nm.

2.3. Gelatin zymography

The enzyme activity of MMP-2 was measured by Gelatin zymography. Briefly, 50 μl of culture supernatant was electrophoresed on 7.5% SDS-PAGE gel, containing 0.1% gelatin (Sigma–Aldrich Co., St. Louis, MO, USA) as the substrate. After electrophoresis, the gel was washed twice with washing buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100. The gel was incubated with incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 1 mM $ZnCl_2$) at 37 °C. For the development of enzyme activity, the gels were stained with Coomassie brilliant blue R-250 and destained in methanol/acetic acid/H_2O. Quantification of MMP activity was performed using the Multi Gauge densitometry program (Fujifilm).

2.4. Western blot

Cell lysates were prepared as described by Yu and Xing, 2006. Protein concentrations were measured by BCA protein assay kit (Bio-Rad, Hercules, CA). Aliquots of proteins were subjected to SDS-PAGE and transferred onto Polyvinylidene fluoride membranes. After blocking with 2% BSA, blots were probed with monoclonal antibodies against PCNA, TGF-β1, TGF-β2, TGF-β3, TβRII, Smad2, Smad3, Smad7 and phosphorylated Smad2 and Smad3 (all from Cell Signaling Technology, Danvers, MA). Secondary antibody was conjugated with horseradish peroxidase; blot was developed with an ECL detection assay (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then stripped and reprobed with antibodies to β-actin to control for protein loading. The intensity of the bands was analyzed by densitometry.

2.5. Quantitative real-time RT-PCR

Total RNA was extracted from atRA-treated cells by the Trizol kit (Gibco/Life Technologies) according to the manufacturer's protocol. cDNA synthesis was performed with Superscript III First-Strand Synthesis System (Invitrogen). The amplification protocol comprised 32 cycles of denaturation for 20 s at 94 °C, annealing for 20 s at 56 °C, and elongation for 30 s at 72 °C. The housekeeping gene GAPDH was used as an internal control, and gene-specific mRNA expression was normalized to GAPDH expression by using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were summarized in Table 1.

2.6. Adenovirus transfer

Cells were seeded onto 6-well plates at a density of 7×10^4 cells/well and maintained in DMEM medium supplemented with 5% FBS. The following day when the cultures were 50–60% confluent, the cells were transfected with expressing vector pCMV-myc without a cDNA insert (empty vector) or with HA-tagged full-length Smad 3 using Effectene transfection reagent (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The control plasmid pRL-CMV was co-transfected to normalize for transfection efficiencies. Twenty hours post-transfection, cells were washed with fresh culture medium and stimulated with 5 ng/ml TGF-β3 (R&D Systems Inc.) or 1.0 mM RA for 24 h. Each condition was analyzed in triplicate and the experiment was performed three times with comparable results.

2.7. Statistical analysis

All the grouped data were evaluated using SPSS13.0 software. One-way analysis of variance followed by Boneferroni/Dunn post hoc multiple comparison tests was used to determine statistical significance between groups. Data are shown as mean ± SEM of four independent experiments. A value of $^*P < 0.01$ was regarded as significant.