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RESEARCH ARTICLE

Immunolocalization of connective tissue growth factor, transforming growth factor-beta1 and phosphorylated-SMAD2/3 during the postnatal tooth development and formation of junctional epithelium

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ABSTRACT

Connective tissue growth factor (CTGF) is a downstream mediator of transforming growth factor-beta 1 (TGF-β_1) and TGF-β_1-induced CTGF expression is regulated through SMAD pathway. However, there is no literature showing the expression of TGF-β_1-SMAD2/3-CTGF signaling pathway during postnatal tooth development and the formation of junctional epithelium (JE). Hence, we aimed to analyze the localization of TGF-β_1, CTGF and phosphorylated SMAD2/3 (p-SMAD2/3) in the developing postnatal rat molars. Wistar rats were killed at postnatal (PN) 0.5, 3.5, 7, 14 and 21 days and the upper jaws were processed for immunohistochemistry. At PN0.5 and PN3.5, weak staining for TGF-β_1 and CTGF was evident in preameloblasts (PA), while moderate to strong staining was seen in odontoblasts (OD), dental papilla (DPL), secretary ameloblasts (SA), preodontoblasts (PO) and polarized odontoblasts (PoO). There was no staining for p-SMAD2/3 in PA, SA, PO and PoO, although strong staining was localized in DPL. OD was initially moderately positive and then negative for p-SMAD2/3. At PN7, intense staining for TGF-β_1 and CTGF was observed in SA, OD, dental pulp (DP) and predentin respectively. p-SMAD2/3 was strongly expressed in DP and moderately expressed in SA and OD. At PN14 and PN21, both reduced enamel epithelium (REE) and JE showed a strong reaction for TGF-β_1 and CTGF. p-SMAD2/3 was intensely and weakly expressed in REE and JE respectively. These data demonstrate that the expression of CTGF, TGF-β_1 and p-SNAD2/3 is tissue-specific and stage-specific, and indicate a regulatory role for a TGF-β_1-SMAD2/3-CTGF signaling pathway in amelogenesis, dentinogenesis and formation of JE.

1. Introduction

Teeth are typical examples of epithelial-mesenchymal organs. The first morphological sign of tooth development is a thickening of the oral epithelium, which subsequently buds into the underlying mesenchyme. The mesenchymal cells condense around the bud, and during the following cap and bell stages, the epithelium undergoes folding morphogenesis resulting in the establishment of the form of the tooth crown (Thesleff and Sharpe, 1997). Many of the molecular mechanisms responsible for the morphogenetic stages of tooth development from initiation through early bell stages have been elucidated. There is less known, however, of the signaling that results in the polarization, differentiation, extracellular matrix production and phenotypic protein expression patterns of the odontoblasts and ameloblasts that produce the structural dentin and enamel.

Junctional epithelium (JE) was originally derived from the reduced enamel epithelium, and will be replaced in time by a JE formed by basal cells originating from the oral gingival epithelium (Shimono et al., 2003; Bosshardt and Lang, 2005). Although the structural and functional characteristics of the JE have been well recognized, the molecular mechanisms leading to the formation of JE are not fully understood. *In situ* hybridization and immunohistochemical analysis by Dias et al. (2005) showed that syndecan-1 was involved in the formation of JE. Recently, efforts to identify the secretome of the epithelial cells responsible for creating tooth enamel have led to the identification of two genes encoding secreted proteins called Amelotin (AMTN) and Odontogenic Ameloblast-Associated (ODAM). Broad expression profiling revealed that both ODAM and AMTN were found not only in the maturation stage of amelogenesis but were also strongly expressed

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in the JE (Nishio et al., 2013; Holcroft and Ganss, 2011). Furthermore, immunohistochemical analysis on integrin $\beta_3^{-/-}$ mice demonstrated that integrin-mediated ODAM was involved in the attachment of JE and ameloblasts to the tooth surface (Lee et al., 2015). These results emphasize the fact that there is a developmental continuum from enamel epithelium to JE. However, relative to the clinical importance of JE in periodontitis pathogenesis, many developmental events of JE await further investigation.

Transforming Growth factor-beta1 (TGF-β_1) is a pleiotropic cytokine with extensive biological activities and is critical for regulating numerous developmental and homeostatic processes, including wound healing (Hongo et al., 2016). TGF-β_1 exerts its biological effects by binding to a cell surface receptor complex of type I and type II receptors. Upon its ligation, the type II receptor phosphorylates the type I receptor (TGFβRI). TGFβRI kinases phosphorylate SMAD2 and SMAD3, which form a transcriptional complex with SMAD4. This complex translocates into the nucleus to initiate the transcription of target genes (Yoshimoto et al., 2015). One of the targets of TGF-β signaling is a cysteine-rich secretory protein CCN2 (also known as connective tissue growth factor, CTGF). CTGF belongs to CCN family, which consists of CCN1/CYR61, CCN2/CTGF, CCN3/NOV, CCN4/WISP-1, CCN5/WISP-2 and CCN6/WISP-3 (Muromachi et al., 2015). CTGF exhibits diverse cellular functions including extracellular matrix production, angiogenesis, normal growth and development of certain tissues, and cell migration and adhesion (Takeuchi et al., 2009). According to a previous report (Pacheco et al., 2008), CTGF and TGF-β signaling components such as TGF-β_1, TGF-β receptor II, SMAD2/3 and SMAD4 were localized in inducer tissues during early stages of tooth development. In addition, Fujita et al. (2012) demonstrated that Smad2 and Bcl-2 are essential components of apoptosis-mediated homeostasis in the JE. However, details of the expression of CTGF, TGF-β_1, phosphorylated SMAD2/3 (p-SMAD2/3) during the cytodifferentiation of ameloblasts, odontoblasts and the development of JE are still unclear.

In the present study, we investigated the immunolocalization of CTGF, TGF-β_1 and p-SMAD2/3 during postnatal tooth development and the formation of JE, which will advance our understanding of specific events critical to amelogenesis, dentinogenesis and dentogingival attachment apparatus.

2. Material and methods

2.1. Preparation of tissue sections and histological staining

In this study, 25 Wistar rats (postnatal 0.5, 3.5, 7, 14, 21 days) were used, following approval from the Animal Ethics Committee, School and Hospital of Stomatology, Wenzhou Medical University. Five Wistar rats in each age group were deeply anesthetized by chloral hydrate and perfusion fixed transcardially with phosphate-buffered formalin solution (10%, pH 7.2, 4 °C). The maxillary molars, together with the surrounding alveolar bone and gingival tissues were block dissected and immersed in the same fixative at 4 °C overnight. After fixation, the maxillae were trimmed into small blocks, decalcified for 3-6 weeks by immersion in EDTA (10%, pH 7.2, 4 °C) solution, dehydrated using a graded ethanol series and embedded in paraffin. Serial 5 μm longitudinal sections were cut in the bucco-lingual plane of the tooth and stained with hematoxylin-eosin for assessment of tissue histology.

2.2. Immunohistochemistry

The polyclonal goat anti-rat p-SMAD2/3 IgG and polyclonal rabbit anti-rat CTGF, TGF-β_1 (Santa Cruz Biotechnology, USA) Igs were used. Sections were deparaffinized in xylene and rehydrated in 100%, 95%, 85%, 70% alcohol, then in distilled water and phosphate-buffered saline (PBS). Enzymatic pretreatment with 0.1% (wt/vol) trypsin (Zhongshan, Beijing, China), for 15 min at 37 °C, was performed to increase accessibility of antibody to the epitopes, after which the sections were washed in PBS and soaked in 3% hydrogen peroxidase solution (Zhongshan) for 15 min to block endogenous peroxidase activity. The sections were then blocked with 5% normal goat serum for the staining of CTGF and TGF-β_1, or with 5% skim milk for the staining of p-SMAD2/3, for 20 min at room temperature, followed by incubation of the primary antibody (CTGF, 1:300; TGF-β_1, 1:250; p-SMAD2/3, 1:40) overnight at 4 °C. Negative controls were obtained by replacing primary antibody with PBS. After three washes in PBS, the sections were incubated with a biotinylated secondary antibody followed by incubation with conjugated streptavidin-peroxidase (Zhongshan) for 20 min, or with polymer helper followed by poly-horseradish peroxidase anti-goat IgG for 20 min (Zhongshan). Specific immunostaining was visualized by incubation with 3,3′-diaminobenzidine tetrachloride solution (Zhongshan) for 3 min and the sections were washed twice with distilled water. Then the sections were counterstained with hematoxylin solution, rinsed in running tap water, dehydrated in a series of ethanol and cleared with xylene. All the sections were viewed and photographically recorded using an Olympus BX50 Microscope (Olympus, Tokyo, Japan).

2.3. Assessment

An assessment of the localization of the antibody staining was made, as described by Matias et al. (2003). The intensity of the staining was determined by 3 oral pathologists who were not related to this study. Immunoreactivity of the antibody in the major tissues and cells was given a score (−, negative; +/−, weakly positive; +, moderately positive; or ++, strongly positive). As this was a descriptive study, statistical analysis of the data was not undertaken.

3. Results

Table 1 summarized the expression of CTGF, TGF-β_1 and p-SMAD2/3 during postnatal tooth development and formation of JE.

3.1. Postnatal (PN) 0.5

The tooth germ was in the presecretory stage. The enamel organ consisted of outer enamel epithelium (OEE), stellate reticulum (SR), stratum intermedium (SI) and inner enamel epithelium (IEE). In the future cusp tips, terminal differentiation of odontoblasts (OD) precedes that of ameloblasts. OD differentiation was marked by elongation, polarization and the secretion of predentin matrix. Preameloblasts (PA) were in direct contact with acellular predentin. In the future fossal areas, the PA was separated from preodontoblasts (PO) by a distinct basement membrane (Fig. 1A–C). Weak staining for CTGF was detected in PA and SI, while CTGF was strongly expressed in SR, OEE, OD and dental papilla (DPL) (Fig. 1D–F). CTGF was strongly immunopositive in oral epithelium, but negative in the capillary endothelium (not shown). Weak to negative staining for TGF-β_1 was found in PA, SI, SR and OEE, whereas intense staining for TGF-β_1 was noted in OD and DPL (Fig. 1G–I). Strong staining for p-SMAD2/3 was localized in OEE, SR and DPL, while PA was not reactive with p-SMAD2/3. Moreover, SI and OD were weakly and moderately stained for p-SMAD2/3 respectively (Fig. 1J–L).