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Immunolocalization of Smad-4 in developing molar roots of alendronate-treated rats

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ABSTRACT

Introduction: During root formation, Smad-4 plays a key role during the epithelial–mesenchymal interactions and the Hertwig's epithelial root sheath (HERS) apical proliferation. The root formation and eruption of rat molars is impeded by alendronate treatment due to the inhibition of bone resortion by this drug. The present study aimed to examine the structures affected in the developing root and immunodetect the presence of Smad-4 in rats treated with alendronate.

Methods: Newborn Wistar rats were daily injected 2.5 mg/kg alendronate (ALN) during 9, 12 and 30 days. The controls (CON) were injected with saline. The maxillae were fixed and embedded in paraffin or Spurr resin. Paraffin sections were incubated in Smad-4 antibody that was labelled with DAB. The ultrathin sections were examined in a transmission electron microscope.

Results: In ALN, a short portion of root dentine was formed; the epithelial diaphragm (ED) and the dental follicle (DF) were disorganized by the contact of bone trabeculae. The (CON) molar roots developed normally. Smad-4 labelling was detected in the cytoplasm of fibroblasts and cementoblasts adjacent to the cementum in CON; in ALN group, few ED cells presented weak immunolabelling. Ultrastructurally, the ED and DF appeared disrupted due to the presence of thin bone trabeculae between its cells. It resulted in the lack of apical proliferation of HERS and, consequently, arrest of root formation.

Conclusion: The immunodetection of Smad-4 in the DF cells of ALN specimens indicates that the signalling for the differentiation of these cells into cementum-forming fibroblasts and cementoblasts occurs, despite the impairment of root elongation.

1. Introduction

The development of periodontium initiates when root formation starts. It is an event initiated by the epithelial proliferation at the cervical loop where the inner and outer enamel epithelia fuse to produce the epithelial diaphragm and the Hertwig's epithelial root sheath (HERS). As HERS cells proliferate apically, complex epithelial–ectomesenchymal interactions occur preceding the formation of root dentine

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and cementum.[1] Among these interactions, the TGF-β/BMP signalling has been demonstrated to play a role during the initiation of periodontium development;[2,3] Smad-4 is a key mediator of the the canonical TGF-β pathway,[4–7] and it has been proven to be crucial during the root development.[3,8] The TGF-β/BMP and their respective receptors build complexes that phosphorylate the Smad proteins, which translocate into the nucleus to regulate the expression of an array of target genes like sonic hedgehog (Shh), which mediate the epithelial–mesenchymal interactions during root development.[3]

The root and periodontium formation occur simultaneously with the intraosseous and preocclusal stages of tooth eruption.[9] Tooth eruption is a process that involves a dynamic remodelling of the bony crypt. Bone resorption by osteoclasts is necessary for providing the space for the developing teeth inside each bony crypt, and also to create the eruptive pathway during the intraosseous stage of tooth eruption, as well as in the remodelling process during the formation of the alveolar bone.[10,11]

Nitrogen-containing bisphosphonates are potent antiresorptive drugs that are widely employed for prevention and treatment of bone diseases such as osteoporosis, Paget's disease of bone and metastatic bone cancer.[12] They are also used in therapy of several paediatric and juvenile bone disorders.[13–15] The administration of sodium alendronate to young rats occasioned the inhibition of tooth eruption and impaired the root formation of molars due to ankylosis at the cervical portion of the tooth germ.[16] More recently, the inhibition of tooth eruption and root formation in zoledronic acid-treated rats has been also reported.[17] The ankylosis between the alveolar bone and the tooth germ observed in the studies above occasions the disruption of the dental follicle and the enamel epithelia, which are crucial structures during tooth eruption and periodontium development.[1,11]

Since the interactions between HERS and ectomesenchymal cells during the dental root development and tooth eruption are still not completely understood, the impairment of this process by alendronate treatment offers an interesting model to verify how such interactions occur when several structures are affected by the drug. We used an experimental model in which sodium alendronate was daily administered to newborn rats from the day of birth until 30 days old.[16,18] The aim of the present study was to analyze the structures affected in the impairment of root formation and periodontal development by alendronate. The immunolabelling of Smad-4 was employed to verify which structures respond to BMP/TGF-β signalling during these processes and whether the impairment of root and periodontium formation is related to the inhibition of this pathway. Additionally, the detection of apoptotic cells in the treated specimens was performed and the fine structure of developing root and periodontium was analyzed by transmission electron microscopy.

2. Materials and methods

2.1. Sodium alendronate treatment

Principles of laboratory animal care (NIH publication 85-23, 1985) and national laws on animal use were observed for the present study, which was authorized by the Ethical Committee for Animal Research of the University of São Paulo, Brazil.

Forty-eight newborn Wistar rats were used in this study. Twenty-four rats were subjected to daily subcutaneous injections of 2.5 mg/kg/day sodium alendronate[16,18,19] since the day of birth to 9, 12 and 30 days old; additional 24 rats were daily injected with sterile saline solution during the same periods as controls. All the alendronate-treated rats were not weaned during the entire study in order to have their nutrition provided maternally.

2.2. Sample obtaining and fixation

On the days cited, eight alendronate-treated and eight control rats were anaesthetised with 2% chloridrate 2-(6,6-xilidine)-5,6-dihydro-4-H-1,3-tiazine (Rompun®) diluted 1:1 in ketamine (Francotar®), 1 ml/kg body weight, decapitated and had their maxillary alveolar processes dissected out and immediately fixed in 0.1% glutaraldehyde and 4% formaldehyde buffered in 0.1 M sodium cacodylate, pH 7.4. Specimens were immersed in a beaker containing 40 ml of fixative solution at room temperature, which was subsequently placed in a Pelco 3440 laboratory microwave oven (Ted Pella, Redding, CA, USA). The temperature probe of the oven was submersed into the fixative and the specimens were then exposed to microwave irradiation at 100% setting for 3 cycles of 5 min, with the temperature programmed to a maximum of 37 °C. After microwave irradiation, specimens were transferred into fresh fixative solution and left submersed overnight at 4 °C.[20] The specimens were decalcified in 4.13% EDTA during 4 weeks.

2.3. Light microscopy

After decalcifying, the specimens from four rats at each time point were dehydrated in crescent concentrations of ethanol and embedded in paraplast. Five-μm thick sections were obtained in a Micron HM360 microtome and stained with haematoxylin and eosin. Coverslips were mounted with entellan and the slides examined in an Olympus BX60 light microscope.

2.4. Immunohistochemical detection of Smad-4

Some sections were left unstained and submitted to immunohistochemical detection of Smad-4. After dewaxing, the sections were heated to 60 °C for 15 min and treated with H_2O_2/methanol solution (1:1) during 20 min. The non-specific binding sites were blocked during 1 h with 10% non-immune swine serum (Dako, Carpinteria, CA, USA) in 1% BSA. Then, they were incubated with the primary antibody (anti-Smad-4, 1:200, Sigma, St. Louis, MO, USA) during 2 h, at room temperature within a humid chamber. After rinsing with buffer, detection was achieved using DAB as substrate (Dako), and nuclei were stained with Harris's haematoxylin. Negative controls were incubated in the absence of primary antibody.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)

Specimens from ALN and CON group were fixed and decalcified and paraffin-embedded as described above.