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Kinetics of gene expression of alkaline phosphatase during healing of alveolar bone in rats

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Abstract

Immunohistochemical studies and molecular biology have enabled us to identify numerous proteins that are involved in the metabolism of bone, and their encoding genes. Among these is alkaline phosphatase (ALP), an enzyme that is responsible for the initiation of mineralisation of the extracellular matrix during alveolar bone repair. To evaluate the gene expression of ALP during this process, we studied nine healthy adult male rats, which had their maxillary central incisors extracted from the right side and were randomly divided into three groups. During three experimental periods, 7 days, 14 days, and 28 days, the alveoli were curetted, the rats killed, and samples analysed by real-time reverse transcription polymerase chain reaction (qRT-PCR). The RNAm that encodes the gene for the synthesis of ALP was expressed during the three periods analysed, but its concentration was significantly increased at 14 and 28 days compared with at 7 days. There was no significant difference between 14 and 28 days ($p = 0.0005$). We conclude that genes related to ALP are expressed throughout the healing process and more intensively during the later periods (14 and 28 days), which coincides with the increased formation of mineralised bone.

Keywords: alveolar bone repair; tooth socket; bone biology; mineralization; alkaline phosphatase; gene expression

Introduction

Scientific predictability of clinical treatments in oral and maxillofacial surgery, implantology, and periodontics is closely related to knowledge of the biopathology of bone. The morphophysiological characterisation and understanding of the dynamics of bony repair are fundamental to the development of better treatments, including oral rehabilitation with bone grafts and implant-supported prostheses.[1]

Experimental studies involving immunohistochemical techniques and molecular biology have allowed identification of several proteins that are involved in the metabolism of bone, among which is alkaline phosphatase (ALP), a specific glycoprotein that is related to the early stages of differentiation of osteoblasts, and seems to be responsible for enabling the growth of hydroxyapatite crystals early in the formation of bone.[2–4]

The extraction of upper incisors in rats is a common experimental model for the evaluation of repair of alveolar bone, which can be divided into three phases with different histological features: exudative (marked by the fibrin synthesis), proliferative marked by formation of granulation tissue), and

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reparative (characterised by the synthesis of collagen, mineralisation, and remodelling).[5–7] The socket is considered healed when it is totally filled by newly-formed bone and the alveolar crest has been remodelled, which happens after 28 days in rats[7] and 64 days in humans.[5]

During alveolar repair it is necessary to increase the neoformation of bone. A cascade of molecular signalling related to the local inflammatory process leads to recruitment, differentiation, and maturation of large numbers of osteoblasts. Osteogenesis is then initiated by stimulus of the transcription of specific mRNA molecules that, in turn, encode the translation of proteins related to bony metabolism.[8] A better assessment of how the genes that encode ALP are expressed during the repair of alveolar bone in animals in homeostatic conditions will provide data that can contribute to future studies, not only to the optimisation of the process, but also for comprehension of healing disorders that arise from systemic changes that affect the skeleton.

The purpose of this study, therefore, was to investigate the dynamics of the expression of the ALP gene during healing of the alveolar bone in healthy rats by quantifying messenger RNA that encodes the specific gene for synthesis of this enzyme using the qRT-PCR technique (Real-time reverse transcription polymerase chain reaction).

Animals and methods

All ethical principles and national laws for the use of laboratory animals were followed, and the study was approved by the Ethics Committee on Animal Experimentation of the Araçatuba Dental School, São Paulo State University "Júlio de Mesquita Filho", Brazil (approval number: 00123-2013).

Animals

Nine adult, male, Wistar rats (*Rattus norvegicus albinus*), body weight 250-300 g, bred in the Animal Care Unit of the Araçatuba Dental School for purposes of education and research, were selected for the study and randomly distributed into three groups of three animals each: three were studied for 7 days, three for 14 days, and three for 28 days.

From the week before, and throughout the whole experimental period, the animals were kept in cages in a stable environment (temperature 22 (2) °C) with a 12 hour light/dark cycle. They were fed on solid crushed chow (Ração Ativada Produtor ®, Anderson & Clayton S.A. – Laboratório Abbot Ltda., São Paulo, SP, Brazil) and had free access to water, except during the 12 hours preoperatively.

Extraction of the upper right central incisor

The operations were done aseptically, and all nine rats had their tooth extraction on the same day. They were weighed and then anaesthetised by intramuscular injection of xylazine hydrochloride (Coopazine ® - Coopers Brazil Ltda, Cotia, SP, Brazil) 0.03 ml/100 g body weight followed by ketamine hydrochloride (Vetaset ® - Fort Dodge Animal Health, Iowa, USA)0.07 ml/100 g body weight.

The anterior portion of the maxilla was wiped with antiseptic (polyvinylpyrrolidone iodine, PVPI 10% Topic - Riodeine ®, Riodeine Chemical Industry, Ltda, São José do Rio Preto, SP, Brazil) before extraction of the upper right incisor with the aid of specially adapted instruments.[5] The gingival fibromucous was then sutured with 4/0 polyglactin 910 (Vicryl), and a dose of pentabiotic (0.1 ml/kg, Fort Dodge Animal Health Ltda, Campinas, São Paulo, Brazil) was given intramuscularly.

Molecular analysis

The samples of bone for analysis were collected at three different stages of repair of the alveolar bone so that we could evaluate the expression of the gene that encodes ALP synthesis. The transcriber mRNA was quantified using qRT-PCR.

Within 7, 14, and 28 days (n = 3 in each group) after the extraction of the right upper incisor, the rats had a further procedure that followed the same preoperative protocol as previously. Fragments of bony tissue were obtained from the middle third of each alveolus with a rongeur, thoroughly washed in phosphate buffered saline, and frozen at 75 °C in liquid nitrogen, after which the rats were killed with an overdose of anaesthetic.

To achieve the highest purity and suitable concentration, total RNA was extracted by ultracentrifugation of the cell sample with Trizol reagent (Life Technologies Invitrogen, Carslbad, CA, USA). After RNA processing for the removal of introns (splicing), capping of the extremity 5′ and polyadenylation of the 3′ end, we obtained mRNA. Each complementary DNA sample (cDNA), which subsequently served as a template for amplification by PCR, was obtained from mRNA 1 μg by enzymatic reverse transcription (M-MLV reverse transcriptase - Promega Corporation, Madison, WI, USA).

The processing of qRT-PCR reactions involved the CFX96 thermocycler (Bio-Rad Laboratories, Philadelphia, PA, USA), the system SybrGreen PCR Master Mix (Applied Byosistems, Warrington, UK) and ALP specific primers (sense ribbon: ACGTGGCTAAGAATGTCATC and antisense ribbon: CTGGTAGGCGATGTCCTTA), designed using the Primer Express® software (Applied Biosystems, Foster City, CA). The standard PCR conditions adopted were 50 °C (2 minutes), 95 °C (10 minutes), and 40 cycles of 95 °C (15 seconds), 60 °C (1 minute), followed by a standard denaturation curve.

The relative gene expression was calculated in reference to the expression of mitochondrial ribosomal proteins and normalised by the gene expression of bone fragments removed