Original

Immunohistochemical Detection of PTHrP and PTH/PTHrP Receptor 1 on the Odontoblastic Reparative Process after Actinomycin D Treatment in Rats

Atsuhiko Kato¹, Masami Suzuki¹, Yayoi Karasawa¹, Tetsuro Sugimoto¹, and Kunio Doi²

¹Safety Assessment Department, Chugai Pharmaceutical Co., Ltd., 1-135 Komakado, Gotemba-shi, Shizuoka 412–8513, Japan
²Department of Veterinary Pathology, Faculty of Agriculture, University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan

Abstract: Parathyroid hormone related peptide (PTHrP) was discovered as a factor causing humoral hypercalcemia of malignancy (HHM). In the previous reports, HHM model rats showed “dentin niche” which is known as an odontoblastic reparative response to cytotoxic agents. In the present study, PTHrP and its receptor (PTHR1) detection patterns were evaluated, during the dentin niche formation process after treatment of a representative cytotoxic agent. Rats were injected with actinomycin D or saline, 9 rats each, and 3 rats from each group were sacrificed on days 1, 3, and 7. The incisors were subjected to immunohistochemical analysis for PTHrP, PTHR1 and ED-1 (macrophage marker), as well as histopathological analysis. On day 1, single cell necrosis and concomitant vesicles that contained necrotic cellular debris were observed. The latter vesicles were positive in the PTHrP, PTHR1 and ED-1 immunostainings. On day 3, depolarization of odontoblasts was observed and hypertrophied pulpal cells were formed osteodentin which stained positively stained for both PTHrP and PTHR1. On day 7, osteodentin had progressed to form dentin niche and the cells which comprised the lesion sustained positive reactions for both PTHrP and PTHR1. These observations suggest that the PTHrP/PTHR1 axis modulates odontoblastic repair and that the modulation might initiate proteins expression in activated macrophages. (J Toxicol Pathol 2005; 18: 33–39)

Key words: PTHrP, PTH/PTHR receptor 1, actinomycin D, tissue repair, odontoblast

Introduction

Parathyroid hormone related peptide (PTHrP) was initially discovered as an essential factor causing humoral hypercalcemia of malignancy (HHM), and together with its receptor (PTH/PTHR receptor 1 or PTHR1), is known to be distributed in several tissues and to function as a modulator of tissue development1,2. For exploration into the nature of HHM and its effect in clinical treatment, several HHM animal models have been developed that successfully manifest symptoms similar to those of HHM patients, including hypercalcemia and HHM-related morphological and functional changes in the bones and kidneys3-9. Our group developed several HHM rodent xenograft models by implantation of human cancer cell lines, which are known to express high levels of PTHrP10, and found one of the models showed odontoblastic lesions as well as other known HHM symptoms11. Interestingly, one of the odontoblastic lesions observed in this model was identical to dentin niche, which is known to consistently develop after administration of a number of cytotoxic agents with different modes of action. Dentin niche is considered to be an odontoblastic repair response to cell damage, because of the consistency and the outcomes of time course observations of its development12-19. PTHrP and/or PTHR1 are known to be expressed in emerging cells during the repair processes in bone, cartilage, and skin20-24, as well as in cells contributing to normal dentinogenesis25-27. In addition, we recently found PTHrP and PTHR1 expression within the cells which comprise the dentine niche in our HHM model rats (unpublished data), which suggests that a sustained level of PTHrP may be essential for induction of the odontoblastic repair process irrespective of the presence of preceding cytotoxicity11,28. However, there is a dearth of information regarding the possible roles of PTHrP and/or PTHR1 during the repair process. In the current report, to clarify the temporal
relationship between the formation of dentin niche and localization of PTHrP or PTHR1-expressing cells, time course observations after odontoblastic cell damage induced by actinomycin D were performed.

Actinomycin D is a cytotoxic agent which suppresses DNA-dependent RNA synthesis through interference with the function of RNA-polymerase29, and the dentin niche formation process after its treatment has been well documented17. Accordingly, we selected this compound as a representative cytotoxic agent to induce dentin niche in the incisors of rats.

Materials and Methods

Animals

Eighteen 5-week-old male Sprague-Dawley rats (Crj:CD(SD)IGS, purchased from Charles River Japan, Inc. Kanagawa, Japan) were housed in sterilized cages in an animal room maintained at a temperature of 24 ± 2°C and a humidity of 55 ± 10%, with 14 to 16 air changes per hour and a 14-hour light and 10-hour dark cycle (lighting from 5:00 to 19:00). The animals were allowed free access to standard rodent chow (CE-2, Clea Japan Inc.) and sterilized water throughout the study.

After one week of acclimatization, 9 rats received a single subcutaneous injection of actinomycin D (AD) (Sigma, Tokyo, Japan) at a dose of 0.375 mg/kg (0.3 mL/100 g body weight: AD group); the remaining 9 rats each received a 1 mL/animal injection of saline (control group). In this report, the day of injection was designated as day 0 and the days of sampling were chosen as days 1, 3, and 7 after administration of AD or vehicle saline. At each sampling point, 3 animals from each group were sacrificed by exsanguination from the abdominal aorta under deep anesthesia with ether.

The experimental protocol and animal care were approved by the Ethics Committee for the Treatment of Laboratory Animals at Chugai Pharmaceutical Co., Ltd., and the experiment was performed in accordance with the animal welfare guidelines of the committee.

Histopathology

Both mandibular incisors and both tibias were removed from all animals; the left side samples were fixed with 20%
neutral buffered formalin and decalcified with ethylenediaminetetraacetic acid·4Na (EDTA·4Na) for 2 weeks at room temperature. After transversal trimming of the apical parts of the incisors and longitudinal trimming of metaphyseal parts of the tibias, the samples were embedded in paraffin using the conventional method and stained with hematoxylin and eosin (HE). The right side samples were fixed with periodate-lysine-paraformaldehyde fixative (PLP fixative, containing 4% paraformaldehyde) at 4°C overnight and decalcified with EDTA·4Na for 1 month at –5°C. After the same trimming that was applied to left side, the samples were embedded in paraffin by the AMeX method and subjected to immunohistochemistry.

For immunohistochemistry, anti-human PTHrP (10 µg/mL) and PTHR1 (8 µg/mL) goat antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-rat monocytes antibody (ED-1; 2 µg/mL) (BMA Biomedicals, Augst, Switzerland) were applied as the primary antibodies and positive reactions were visualized by the LSAB method with a diaminobenzidine reaction. As negative controls for immunohistochemistry, normal goat serum (NGS) and normal mouse serum (NMS) were applied as substitutes for both primary anti-PTHrP and -PTHR1 antibodies, and for ED-1 antibody, respectively. For validation of positive reactions, the tibial metaphyseal area of the control group was stained with the aforementioned antibodies and positive reactions were compared with the previously reported data.

To analyze histopathological features in HE-stained sections as well as the immunohistochemical detection patterns, odontoblasts were further classified as mesenchymal cells, pre-odontoblasts, and columnar odontoblasts, based on previous reports (Fig. 1).

### Results

**Histopathological changes in HE sections of the AD group**

In all samples from the AD group, histological changes were observed in the odontoblastic and adjacent pulpal cells, but not in any other dentinogenic cells such as ameloblasts or cementoblasts.

In the day 1 incisor samples of the AD group, single cell necrosis of the mesenchymal cells of the apical end of the dentin and the adjacent pulpal cells were observed with slight edema in the surrounding pulpal matrix. A small population of the cells showed a tendency of minor decrease of cell densities, but this was not a general finding of these cell populations. Vesicles which contained cell debris from single necrotic cells were also observed within the matrix (cell debris containing vesicle, CDCV) (Fig. 2, AD, Day 1). The pre-odontoblasts, columnar odontoblasts, and the corresponding adjacent pulpal cells remained intact (Table 1).

On day 3, the cell density of mesenchymal cells and adjacent pulpal cells had decreased due to the edematous exudation of this area. Single cell necrosis had subsided and CDCVs were absent at this time point. In pre-odontoblasts and adjacent pulpal cells, the decrease in cell density was more severe than that of mesenchymal cells. In severely affected cases, the pre-odontoblastic layer was devoid of pre-odontoblasts and pulp cells and was occupied by acellular edematous fluid (Fig. 2, AD, Day 3, right). In the transitional zone from pre-odontoblasts to columnar odontoblasts, the pre-odontoblasts were depolarized and adjacent pulpal cells were hypertrophied. The pre-odontoblasts were surrounded by dentin matrix (osteodentin) (Fig. 2, AD, Day 3, left). The columnar odontoblasts and adjacent pulpal cells remained intact (Table 1).

On day 7, the osteodentins had formed dentin niche as a sharply demarcated recessed area within the surrounding dentin. The inner surface of the dentin niche was covered by odontoblastic cells which were more cuboidal in morphology than the surrounding normal columnar odontoblasts (Fig. 2, AD, Day 7). There were no obvious lesions in the mesenchymal cells, pre-odontoblasts and pulpal cells, and normal structure was regained at this time point (Table 1).
**Immunohistochemistry**

In the control group, mesenchymal cells and adjacent pulpal cells were weakly positive for both PTHrP and PTHR1. Pre-odontoblasts were positive for both proteins, while the adjacent pulpal cells were weakly positive. Columnar odontoblast and adjacent pulpal cells showed positive reactions to both proteins. In the ED-1 stained section, positive reactions were sporadically observed in the relatively small spindle cells within the pulpal tissue (Fig. 3).

In the day 1 samples from the AD group, the intensity of the positive reactions for PTHrP and PTHR1 immunostaining in the mesenchymal cells and pulpal cells were identical or less than those of the control group. For CDCV, the membrane on the vesicles was not only positive for both PTHrP and PTHR1, but it was also positive for ED-1 (Fig. 3).

On day 3, both depolarized odontoblasts and hypertrophied pulpal cells, which composed osteodentin, showed positive reactions for both PTHrP and PTHR1, and they were more intense than those of the corresponding cells of the control group (Fig. 3).

On day 7, both depolarized odontoblasts and hypertrophied pulpal cells, which composed osteodentin, showed positive reactions for both PTHrP and PTHR1, and they were more intense than those of the control group (Fig. 3).

NGS (negative control for both PTHrP and PTHR1) and NMS (negative control for ED-1) did not produce any specific positive reactions in any section. The distribution patterns of the PTHrP and PTHR1 positive chondrocytes in the tibial epiphyseal growth plates in the control group were identical to those of previously reported23. ED-1 positive reactions were also detected in osteoclasts of the tibial sections as previously reported36. From these results, we concluded that the immunostains for PTHrP, PTHR1, and ED-1 were valid.

**Discussion**

The incisors of rodents are known to grow throughout life, and are composed of a dentin core covered by enamel and cementum at the labial and lingual surface, respectively. They enclose incisor pulp, and within the pulp, mesenchymal cells around the apical end of the incisor differentiate into odontoblasts, which start secreting dentin and moving to the incisal direction37. Accordingly, to clarify the mechanism of chemically-induced odontoblastic changes, temporal observation which covers the differentiation of these cells, is required.

In the current study, the odontoblastic repair process after cell damage induced by a cytotoxic agent was successfully reproduced (Fig. 4). On day 1, actively replicating mesenchymal cells and surrounding pulpal cells were concomitant with their single cell necrosis as well as slight edema. Moule et al. first described the emergence of CDCV on 1 day after chemical insult and suggested they were macrophage from electron microscopical features and positive reactions to acid phosphatase staining17. We also observed the appearance of CDCV with the same timing during the odontoblastic repair process and their ED-1 positive reactions are a signature of macrophage lineage. On day 3, odontoblastic change had moved to the pre-odontoblastic area. A decrease in cell density was evident and was associated with severe edema. In the transitional zone near the columnar odontoblasts, depolarized pre-odontoblasts were incorporated within dentin which had presumably been secreted by them and/or the hypertrophied surrounding pulpal cells. On day 7, the lesions moved further toward the columnar odontoblastic area, and formed fully bloomed dentin niche with the histopathological character of sharply demarcated recessed areas of dentin as defined in previous reports12–19. Based on this morphological sequence from the onset of cell damage to dentin niche formation and the fact that many different types of cytotoxic agents with different modes of action can consistently induce dentin niche, the lesion is considered to be the repair response of odontoblasts to cell damage12–19. In addition, previous autoradiographic investigations have shown that cuboidal odontoblastic cells are derived from surviving odontoblasts and hypertrophic pulpal cells, and they are considered to be a signature of regeneration of the odontoblastic layer14,19.

As shown by PTHrP and PTHR1 immunostaining, the cells comprising dentin niche in the present study were consistently positive, along with the macrophages (CDCV). Based on these facts, it is possible to deduced that the cells which committed to the repair process, such as surviving odontoblasts or surrounding pulpal cells, expressed both...
PTHrP and PTHR1. In the repair process of bone, cartilage and skin, expressions of both PTHrP and PTHR1 have been demonstrated, and their function is considered to be a modulator of the process. In our previously reported HHM model, dentin niche was induced by sustained high levels of PTHrP for long duration without any preceding odontoblastic cytotoxic damage, and the administration of an antibody, which neutralizes the PTHrP effect, was able to prevent its formation. Taken together with the knowledge accumulated other tissues and the HHM model, it is plausible to consider that the PTHrP/PTHR1 axis may be the modulator of the odontoblastic repair process as well.

It is also known that PTHrP expressing macrophages emerge in the early phase of the repair process in the skin. Macrophages have been shown to increase PTHrP expression level after stimulation with IL-1 and phorbol myristate acetate in vitro, suggesting the importance of PTHrP-expressing activated macrophages in the repair process. Taking this information into consideration, it is possible to postulate that the emergence of PTHrP and PTHR1 expressing macrophages at an early phase of odontoblastic repair is related to the onset of the process.

In conclusion, as is the case in other tissue repair processes, we suggest that the PTHrP/PTHR1 axis modulates the odontoblastic repair process and the initiation of this process might be related to PTHrP and PTHR1 expressions in cell debris containing activated macrophages.

References