An immunohistochemical study of the expression of heat-shock protein-25 and cell proliferation in the dental pulp and enamel organ during odontogenesis in rat molars

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Summary

Objectives: The aim of this study is to clarify the functional significance of heat-shock protein (HSP)-25 during tooth development.

Design: We compared the expression of HSP-25 in the dental epithelial and mesenchymal cells with their proliferative activity during odontogenesis in rat molars on postnatal Days 1–100 by immunohistochemistry using anti-HSP-25 and anti-5-bromo-2'-deoxyuridine (BrdU) for cell proliferation assay.

Results: On Day one, BrdU-immunoreactive cells were densely located in the inner enamel epithelium in the cervical loop and inter-cusped areas and the dental pulp adjacent to them, whereas HSP-25-immunoractivity (IR) was restricted to the cusped area where odontoblasts and ameloblasts had already differentiated. Subsequently, BrdU-IR shifted in the apical direction to be localized around Hertwig's epithelial root sheath during Days 5–30, never overlapping with concomitantly apically-shifted HSP-25-IR. On Days 60–100, BrdU-immunoreactive cells were hardly recognizable in the dental pulp, where HSP-25-IR was exclusively localized in the odontoblast layer. Furthermore, the odontoblast- and ameloblast-lineage cells exhibited two steps in the expression of HSP-25 throughout the postnatal stages: first, dental epithelial and pulpal mesenchymal cells showed a weak IR for HSP-25 after the cessation of their proliferative activity, and subsequently odontoblasts and ameloblasts consistently expressed an intense HSP-25-IR.

Conclusion: Odontoblast- and ameloblast-lineage cells acquire HSP-25-IR after they complete their cell division, suggesting that this protein acts as a switch between cell proliferation and differentiation during tooth development. The consistent expression of HSP-25-IR in the formative cells may be involved in the maintenance of their functional integrity.

KEYWORDS: Cell Proliferation; Cell Differentiation; Heat-Shock Proteins; Immunohistochemistry; Molar; Odontogenesis; Rats

Introduction

Heat-shock proteins (HSPs), or stress proteins, are evolutionarily ancient and highly conserved proteins, whose synthesis is transiently induced in the cells in response to the many physiological and environmental stresses they encounter. HSPs may be divided into five major families on the basis of their molecular weights: HSP-110, HSP-90, HSP-70, HSP-60, and small HSP families. HSP-25 (a homologue of human HSP-27; the term HSP-25 is applied to its homologue in this paper) belongs to a family of the small HSPs, [1] which functions not only as a molecular chaperone, [2] but also a suppresser of apoptosis, [3, 4] an inhibitor of actin polymerization[5] and a modulator of actin dynamics. [6, 7] Based on the evidence presented in previous studies, it is suggested that HSP-25 has two distinct roles under stressful conditions: (1) to maintain the normal function of the cell through stabilization of the cytoskeleton and by facilitating the repair or removal of damaged proteins, and (2) to prevent apoptosis by interfering with caspase activation. [8] Recently, HSP-25 has been reported to be expressed in the developing process of various tissues and organs including muscle, [9] nerve, [10] cartilage, [11] temporomandibular joint, [12] skin, [13] and tooth. [14] In the process of tooth development, ameloblasts and odontoblasts express HSP-25 consistently, whereas the expression of HSP-25 in pulpal mesenchymal cells is transient. [14-16] HSP-25 expression is also localized in the periodontal tissue including the cementoblasts and the epithelial cell rests of Malassez in rat molars. [17] However, the functional significance of

HSP-25 in the tooth is still unknown, although it has been reported that this protein may play possible roles such as the chaperone function related to secretory activity and the reinforcement of the cytoskeleton under stressful conditions including cell movement or conformational changes during odontogenesis. [14-17]

Investigation concerning cell kinetics in the process of mammalian tooth development had previously depended on autoradiographic methods using ^{3}H-thymidine (^{3}H-TdR). The previous studies using ^{3}H-TdR labeling[18-22] reported that numerous proliferating cells exist in the inner enamel epithelium, stratum intermedium, and Hertwig's epithelial root sheath (HERS) during amelogenesis, whereas a few proliferative cells occur in the other components of the enamel organ. The dental pulp and periodontal tissue also contain numerous proliferating cells in the process of tooth development. Recently, 5-bromo-2'-deoxyuridine (BrdU) has been widely utilized for the investigation of cell proliferation, because BrdU is incorporated into the nucleus during the synthetic phase of cell division in the same manner as ^{3}H-TdR. [23-26] Furthermore, non-proliferative cells are exclusively localized in the enamel knot which plays a pivotal function in the regulation of morphogenesis during tooth development. [25, 27] Since recent study has demonstrated that HSP-25 is involved in the differentiation of rat olfactory neurons[28] and keratinocytes, [29, 30] it is plausible to consider that HSP-25 is involved in the differentiation of odontoblast- and ameloblast-lineage cells during odontogenesis. Thus, we focused on the spatial relation

between the expression of HSP-25 and cell division during the postnatal stages of tooth development in rat molars by double immunohistochemistry using antibodies for both HSP-25 and BrdU.

Materials and methods

All experiments were performed following the guidelines of the Niigata University Intramural Animal Use and Care Committee. Thirty five Wistar rats (postnatal Day one, five, 10, 15, 30, 60, and 100; five animals per group) were intraperitoneally injected with BrdU (150 mg/kg) two h before the fixation, and transcardially perfused with physiological saline followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) under deep anesthesia by an intraperitoneal injection of chloral hydrate (400 mg/kg). The heads or maxillae including molar teeth were removed *en bloc* and immersed in the same fixative for 24 h. Following decalcification of the tissues with 4.13% EDTA at 4°C, the specimens were embedded in paraffin. Sagittal sections were cut at a thickness of 5 μm, and mounted on MAS-coated glass slides.

For the immuno-peroxidase procedure, the sections were processed for the avidin-biotin peroxidase complex (ABC) method using anti-HSP-25-polyclonal antibody (StressGen Biotechnologies Corp, Victoria, BC, Canada) and biotinylated BrdU-monoclonal antibody (Calbiochem-Novabiochem Corp, San Diego, California, USA). Our immunohistochemical protocol for HSP-25 is shown in our previous report, [14] although the antigen-antibody reaction sites were made visible by 5-bromo 4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) in this study. We used the BrdU immunohistochemistry System (Calbiochem-Novabiochem Corp) to detect

BrdU-incorporated cells according to the instruction provided by the company. The immunostained sections were counter-stained with 0.5% methylene green. Immunohistochemical controls were performed by replacing the primary antibody with non-immune serum or phosphate buffered saline. These immunostained sections did not contain any specific immunoreaction.

Results

The expression of HSP-25- and BrdU-IR varied in the dental pulp and enamel organ according to the stages of odontogenesis (Figs. 1–7).

Dentinogenesis

On postnatal Day one, the cusped region of the dental pulp showed HSP-25-IR, whereas BrdU-immunoreactive cells were distributed throughout the dental pulp, being densely located in both the cervical loop and inter-cusped areas (Fig. 1a, b). Preodontoblasts showed weak HSP-25-IR, but odontoblasts and subodontoblastic mesenchymal cells showed intense HSP-25-IR, being negative for BrdU-IR (Fig. 1b, c).

On Day five, pulpal mesenchymal cells in the cusped area showed weak HSP-25-IR in contrast to the intense IR in the odontoblasts, and there were few BrdU-immunoreactive cells (Fig. 2a, c). BrdU-immunoreactive cells were densely localized in the apical side of the dental pulp, being situated beneath the HSP-25-immunoreactive region (Fig. 2a, b).

On Day 10, odontoblasts and the other mesenchymal cells in the dental pulp showed HSP-25-IR. BrdU-immunoreactive cells were densely localized in the pulpal mesenchyme beneath the HERS at the apical side of the dental pulp (Fig. 3a, b).

On Day 15, the weak HSP-25-immunoreaction in the dental pulp shifted toward the root pulp, whereas the odontoblasts continued to show intense HSP-25-IR (Fig. 4a). BrdU-immunoreactive cells occurred in the apical region of the root pulp. The relation

between HSP-25- and BrdU-immunoreactive cells was similar to that in the previous stage (Fig. 4b), although BrdU-immunoreactive cells increased in number in the periodontal tissue (Fig. 4a).

On Day 30, beside the intense HSP-25-IR in the odontoblasts, mesenchymal cells in the apical root pulp exhibited HSP-25-IR in contrast to negative IR in the coronal pulp (Fig. 5a). BrdU-immunoreactive cells were present in the apical root pulp, being localized around HERS as seen in the previous stages (Days 10–15), although they decreased in number and area in the dental pulp (Fig. 5b).

After Days 60–100, the expression of HSP-25-IR was exclusively restricted to the odontoblasts. BrdU-immunoreactive cells were hardly recognizable in the dental pulp (Fig. 6a, b).

Amelogenesis

On Day one, BrdU-immunoreactive cells were densely located in the inner enamel epithelium and the stratum intermedium at the cervical loop and inter-cusped regions of the enamel organ, being negative for HSP-25-IR (Fig.1a, b). The preameloblasts showed weak HSP-25-IR, and the ameloblasts were gradually increased in the intensity of HSP-25-IR, whereas both cell types were negative for BrdU-IR (Fig. 1c).

During from Days five to 10 (secretory to maturation stage), the ameloblasts increased in the intensity of HSP-25-IR according to the advance of amelogenesis. BrdU–immunoreactive cells remained in the cervical loop and HERS, which were negative for HSP-25-IR (Figs. 2a, 3a).

On Day 15, the ameloblasts consistently showed intense HSP-25-IR (Fig. 4a), and the epithelium surrounding the cusped tip contained both numerous BrdU-immunoreactive cells concomitantly with HSP-25-positive cells (Fig. 4c). In the areas where the oral epithelial cells connected with the epithelium surrounding the cusped tip, HSP-25-positive cells were increased in number and few BrdU-immunoreactive cells were recognizable (Fig. 4d).

Figure 7 summarizes the spatiotemporal relation between the expression of HSP-25- and BrdU-IR in the dental pulp and enamel organ during odontogenesis.

Discussion

The present study confirmed the chronological changes in the expression of HSP-25-IR in the dental pulp and enamel organ during odontogenesis, which was identical to those in the previous reports. [14-16] Furthermore, the present double immunohistochemical approach using both anti-HSP-25- and anti-BrdU-antibodies was able to demonstrate the precise relation between the expression of HSP-25-IR in the cells and their proliferative activity.

Cell proliferative kinetics is closely related to tooth morphogenesis and differentiation. The previous reports demonstrated that the high cell proliferative activity is involved in tooth morphogenesis including the cusp formation and rapid increase in volume of the tooth germ, and that the dental papilla cells begin to differentiate into the odontoblasts to produce dentin matrix after the cessation of their proliferative activity. [22, 31] The odontoblast- and ameloblast-lineage cells become polarized and secrete dentin or enamel matrix after the withdrawal from the cell cycle. [32-34] The present labeling experiment is the first report to clearly exhibit the chronological changes in the distribution pattern of proliferating cells during odontogenesis after birth including dentinogenesis, amelogenesis, root formation, tooth eruption, and occlusion in the teeth with the limited growth: the polarized and matrix-producing cells lacked their proliferative activity, and the dividing cells were densely located near the HERS during the postnatal Days 10–15 to be reduced sharply from Days 15–30, and being identical to the findings in the previous studies. [18, 21] Furthermore, this

study demonstrated that cell proliferative activity in the epithelium surrounding the cusped tip of the tooth was increased before tooth eruption as represented by Fig. 4c. The increased rate of cellular proliferation may confer a rapid cell supply for the connection between the reduced enamel epithelium and oral epithelium at the onset of tooth eruption.

It is noteworthy that numerous BrdU-immunoreactive areas do not overlap with HSP-25-immunoreactive areas in the dental pulp throughout odontogenesis, and that the distribution of proliferating cells and the expression of HSP-25-IR shift while maintaining the spatial relation between them from the coronal to apical root region in the dental pulp, suggesting that BrdU-immunoreaction always precedes HSP-25-IR. Furthermore, the odontoblast- and ameloblast-lineage cells exhibit two steps in the expression of HSP-25 throughout the postnatal stages: first, the cells with polarity (preodontoblasts and preameloblasts) show a weak IR for HSP-25 after the cessation of their proliferative activity, and subsequently odontoblasts and ameloblasts consistently express an intense HSP-25-IR. Mehlen et al[4] reported the accumulation of HSP-25 at the early stage of differentiation occurring concomitantly with a decreased rate of cellular proliferation, and concluded that HSP-25 has the role of a switch between cell division and differentiation in embryonic stem cells. HSP-25 has also been reported to control the two steps in the differentiation of keratinocytes.[30] These data support our hypothesis that the expression of HSP-25 is involved in the cell differentiation in the process of tooth development. The expression

pattern of p21, a cyclin-dependent kinase inhibitor, is similar to that of HSP-25 during mouse embryogenesis, [35] reinforcing our hypothesis because p21 protein functions as an inducible growth inhibitor that contributes to cell cycle exit and differentiation. The involvement of HSP-25-IR in the cell differentiation could be applied to the regeneration of the odontoblasts in the pulpal healing process after tooth injuries such as cavity reparation and tooth replantation: the tooth injuries cause the degeneration of the afflicted odontoblast layer to result in the loss of HSP-25-immunoreactions, and subsequently newly differentiated odontoblasts acquire HSP-25-IR. [36, 37] Furthermore, the expression of HSP-25 in the neoepidermis during wound healing is also related to the differentiation of keratinocytes. [38]

In conclusion, developed and developing odontoblast- and ameloblast-lineage cells begin to transiently express a weak HSP-25-IR after the completion of their proliferation, and the formative cells (odontoblasts and ameloblasts) continue to express an intense HSP-25-IR during odontogenesis. Thus, HSP-25-IR is suggested to act as a switch between cell proliferation and differentiation during tooth development. The terminal expression of this protein may be necessary for the formative cells to maintain the cell integrity during odontogenesis and even after the completion of root formation.

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Figure legends

Figure 1 Double immunoreactivity (IR) for HSP-25 and BrdU on postnatal Day one (early dentinogenesis and amelogenesis). **a.** The cusped areas of the dental pulp show intense HSP-25-IR (arrows), whereas BrdU-immunoreactive cells are distributed throughout the enamel organ and dental pulp, and are especially densely localized in both the cervical loop and inter-cusped areas (open arrows). Scale bar = 500 μm. **b.** A higher magnification of the boxed area (marked with "b") in **a**. Numerous BrdU-immunoreactive cells are observed in the enamel organ including inner enamel epithelium and stratum intermedium, and the opposed pulpal mesenchymal cells (MC), but are negative IR for HSP-25. Preodontoblasts (POB) begin to have cell polarity and express weak HSP-25-IR. Scale bar = 50 μm. **c.** A higher magnification of the boxed area (marked with "c") in **a**. Preameloblasts (PAB) show weak IR for HSP-25 and ameloblasts are increased in the intensity of IR for HSP-25 in their distal cytoplasm. The odontoblasts (OB) and subodontoblastic mesenchymal cells show intense HSP-25-IR (arrows), being negative IR for BrdU. BrdU-immunoreactive cells are localized in the subodontoblastic layer (arrowheads). Scale bar = 50 μm. AB: ameloblasts, DP: dental pulp, IEE: inner enamel epithelium, PD: predentin, SI: stratum intermedium, SR: stellate reticulum.

Figure 2 Double IR for HSP-25 and BrdU on postnatal Day five (secretory stage of amelogenesis). **a.** The odontoblasts and ameloblasts show intense HSP-25-IR, whereas the other dental pulp cells show weak IR. BrdU-immunoreactive pulpal cells are mainly distributed in the apical part of the dental pulp (arrows). Scale bar = 500 μm. **b.** A higher magnification of the boxed area (marked with "b") in **a**. BrdU-IR is observed beneath the intense HSP-25-immunoreactive areas in the dental pulp. Scale bar = 50 μm. **c.** A higher magnification of the boxed area (marked with "c") in **a**. In addition to intense HSP-25-immunopositive odontoblasts, the other pulpal mesenchymal cells in the cusped area show weak HSP-25-IR, whereas few BrdU-immunoreactive cells are found there. Scale bar = 50 μm. AB: ameloblasts, DP: dental pulp, OB: odontoblasts.

Figure 3 Double IR for HSP-25 and BrdU on postnatal Day 10 (maturation stage of amelogenesis). **a.** The ameloblasts have increased in the intensity of HSP-25-IR, and the immunoreaction pattern for both HSP-25 and BrdU in other cells is almost similar to that in the former stage. Scale bar = 500 μm. **b.** A higher magnification of the boxed area (marked with "b") in **a**. Numerous BrdU-immunoreactive cells are localized in the pulpal mesenchyme beneath Hertwig's epithelial root sheath (HERS). Scale bar = 50 μm. AB: ameloblasts, D: dentin, DP: dental pulp, E: enamel, OB: odontoblasts.

Figure 4 Double IR for HSP-25 and BrdU on postnatal Day 15 (commencement stage of root formation). **a.** The weak HSP-25-immunoreactive area in the dental pulp has shifted

toward the root pulp, although ameloblasts and odontoblasts continue to show intense HSP-25-IR. BrdU-IR is mainly localized in the apical area of dental pulp and the periodontal tissue. Scale bar = 500 μm. **b.** A higher magnification of the boxed area (marked with "b") in **a**. BrdU-immunoreactive pulpal mesenchymal cells are distributed beneath HERS (arrowheads), where some epithelial cells show BrdU-IR. Scale bar = 50 μm. **c.** A higher magnification of the boxed area (marked with "c") in **a**. HSP-25-IR is recognizable in the epithelial cells (arrows) beneath the ameloblasts showing intense HSP-25-IR in the area near the cusped tip, and numerous BrdU-immunoreactive cells are also distributed near these HSP-25-positive cells. Scale bar = 50 μm. **d.** A higher magnification of the boxed area (marked with "d") in **a**. The oral epithelial cells are continuous with the epithelium surrounding the cusped tip, and both cells contain HSP-25-positive cells and few BrdU-immunoreactive cells. Scale bar = 50 μm. AB: ameloblasts, D: dentin, DP: dental pulp, E: enamel space, OB: odontoblasts, OE: oral epithelium.

Figure 5 Double IR for HSP-25 and BrdU on postnatal Day 30 (commencement stage of occlusion). **a.** The coronal odontoblasts continue to show their intense HSP-25-IR, whereas the odontoblasts in the root and floor pulp show weak or negative IR. The dental pulp shows negative IR for BrdU except for the root apex. Scale bar = 500 μm. **b.** A higher magnification of the boxed area (marked with "b") in **a**.

BrdU-immunoreactive mesenchymal cells (arrowheads) are near the intense HSP-25-immunoreactive area. Note the BrdU-immunoreactive epithelial cells in the HERS. Scale bar = 50 μm. D: dentin, DP: dental pulp, OB: odontoblasts.

Figure 6 Double IR for HSP-25 and BrdU on postnatal Day 60 (a) and 100 (b) (completion stage of root formation). All odontoblasts (OB) in the coronal, root and floor pulp increase in HSP-25-IR from Days 60 to 100, and BrdU-IR is hardly recognizable in the dental pulp (DP). Scale bar = 500 μm. D: dentin.

Figure 7 Schematic illustrations of the relation between HSP-25- and BrdU-IR in the enamel organ, dental pulp and periodontal tissue during odontogenesis from postnatal Days one to 100. BrdU-IR is colored in red, whereas intense HSP-25-IR is colored in blue, and the weak example is light blue. BrdU-immunoreactive cells are exclusively distributed in the negative-immunoreactive area for HSP-25.