Transient Expression of Heat Shock Protein (Hsp) 25 in the Dental Pulp and Enamel Organ during Odontogenesis in the Rat Incisor*

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Summary. The expression of heat shock protein (Hsp) 25 during odontogenesis in the dental pulp and enamel organ of rat incisors was investigated by immunocytochemistry and confocal microscopy. In the process of dentin formation, immature odontoblasts first exhibited Hsp 25-immunoreactivity, and increased in immunointensity with the advance of their differentiation. In the dental pulp, in contrast, intense immunoreaction in the mesenchymal cells became weak or negative in parallel with the progress of cell differentiation. The immunoreaction for Hsp 25 in the enamel organ revealed a characteristic stage-related alteration during amelogenesis. In secretory ameloblasts, the immunoreaction for Hsp 25 was found throughout their cell bodies, intense reactivity being located near the proximal and distal terminal webs. At the maturation stage, ruffled ameloblasts (RA) consistently showed Hsp 25-immunoreactivity throughout the cell bodies, whereas smooth-ended ameloblasts (SA) lacking a ruffled border were weak in immunoreaction at the distal cytoplasm. Other cellular elements of the enamel organ were negative. The subcellular localization of Hsp 25-immunoreactivity in this study appeared essentially identical to that of actin filaments as demonstrated by confocal microscopy using rhodamine-labeled phalloidin. These immunocytochemical data suggest that the Hsp 25 molecule is involved in reinforcement of the cell layer following cell movement during odontogenesis and in the formation and maintenance of the ruffled border of RA.

Heat shock and other deleterious environmental and pathophysiologic stresses induce an up-regulation in the synthesis of heat shock proteins (Hsps). These

proteins are thought to enable the cell to survive and recover from stressful conditions. Hsps are largely divided into two groups of large and small Hsps, according to their molecular weights. Since murine Hsp 25, a homologue of human and rat Hsp 27/28, is one of the small Hsps (20–30 kDa), the term Hsp 25 is applied to its homologues in this paper. Hsp 25 is found in adult human cells such as the breast, uterus, cervix, placenta, skin, and platelets (for reviews, see CIOCCA, 1993; ARRIGO and PRÉVILLE, 1999). Hsp 25 expression serves as a protection against cell necrosis induced by stimuli such as hyperthermia (LANDRY et al., 1989; LAVOIE et al., 1993a), anti-cancerous drugs (HUOT et al., 1991), oxidative stress (MEHLEN et al., 1995, 1996a), metabolic poisons (WU and WELSH, 1996), and inflammatory cytokines (MEHLEN et al., 1995). Hsp 25 also works as a molecular chaperone (JAKOB et al., 1993), or enzyme modulating actin dynamics (LAVOIE et al., 1993b; HUOT et al., 1996) to protect cells suffering stressful conditions. Moreover, Hsp 25 is involved in the suppression of apoptosis (MEHLEN et al., 1996b, 1997; SAMALI and COTTER, 1996).

The transient expression of Hsp 25 has been reported during development and cell differentiation (ARRIGO and PRÉVILLE, 1999): Mammalian Hsp 25 is expressed and/or phosphorylated during the early differentiation of several mammalian cells including embryonal carcinoma and stem cells (STAHL et al., 1992), normal B and B lymphoma cells (SPECTOR et al., 1992), osteoblasts (SHAKOORI et al., 1992), promyelocytic leukemia cells (SHAKOORI et al., 1992; SPECTOR et al., 1993), and normal T cells (HANASH et al., 1993).

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Fig. 1. Overview of Hsp 25-immunoreactivity (IR) in transverse sections of the incisor teeth in resin blocks at different stages of odontogenesis. The remaining blocks (20–30 μm in thickness) after the preparation of semithin sections are directly observed under transmitted light. **a.** Early secretory stage of amelogenesis (near the apical end). The section shows strong IR in the dental pulp, weak IR in the enamel organ (arrows), and the absence of IR in the periodontal ligament. **b.** Secretory stage of amelogenesis. The section shows strong IR in the dental pulp and ameloblasts, and the absence of IR in the periodontal ligament. **c.** Maturation stage of amelogenesis. In the dental pulp, intense immunoreactions are exclusively localized in the odontoblasts (OB). Ameloblasts basically show immunopositive reactions, and the vascular elements of the periodontal ligament become positive in immunoreaction (arrowheads). **d.** Pigmentation stage of amelogenesis (near the incisal end). In addition to IR in the odontoblasts and ameloblasts, the periodontal ligament represents IR in certain fibroblasts and vascular elements. The left side of the figure shows the labial side; right, the lingual side. AB: ameloblasts, DP: dental pulp, E: enamel or enamel space, *: periodontal ligament. a–d: $\times 40$

Therefore, it was determined that a definitive clarification of the exact Hsp 25 expression in the respective organ during organogenesis would greatly promote the understanding of development and cell differentiation.

In oral tissue, the dental pulp of the adult human third molar has been reported to contain Hsp 25-immunoreactive cells such as odontoblasts, pulp fibroblasts, and smooth muscle and endothelial cells of vascular walls (SENS et al., 1996). However, the functional significance of the expression of Hsp 25 in the dental pulp has remained undetermined. Furthermore, no information has been available on its expression in the enamel organ.

This study aims to elucidate the role of Hsp 25 in odontogenesis by analyzing its expression in the dental pulp and enamel organ during dentinogenesis and amelogenesis by the use of immunocytochemistry. In our preliminary examinations, we noticed a resemblance between the expression pattern of Hsp 25 and that of actin filaments in the odontoblasts and ameloblasts. Thus, the distribution of actin filaments was also demonstrated in confocal microscopy using rhodamine-labeled phalloidin. We suggest here that Hsp 25 expression in the dental pulp and enamel organ correlates well with the differentiation and/or proper functioning of the odontoblasts and ameloblasts.

Fig. 2. Hsp 25-IR in the dental pulp and enamel organ of the incisor teeth in resin blocks at different stages of odontogenesis. **a.** Higher magnification of the dental pulp in Figure 1a. The section shows strong IR in the dental pulp and the absence of IR in the periodontal ligament (PDL). The subodontoblastic layer displays more intense IR than the odontoblasts. **b.** Higher magnification of the dental pulp in Figure 1c. Intense immunoreactions are exclusively localized in the odontoblasts. **c.** Higher magnification of the dental pulp in Figure 1c. The labial side shows strong IR in both the odontoblasts and subodontoblastic layer. **d.** Higher magnification of the enamel organ in Figure 1a. At the early secretory stage, the ameloblasts show weak IR in the mesial and distal end of cell bodies, in contrast with the intense IR in the odontoblasts. The cells of the stratum intermedium (SI) exhibit moderate IR in their cytoplasm. **e.** Higher magnification of the enamel organ in Figure 1b. At the secretory stage, the ameloblasts show strong IR throughout the cell bodies. The other cellular elements of the enamel organ including SI reveal an absence of IR. **f.** Higher magnification of the enamel organ in Figure 1c. The enamel organ shows strong IR in the cell bodies of RA, weak IR in SA, and the absence of IR in the papillary layer cells (PC). AB ameloblasts, D dentin, DP dental pulp, E enamel or enamel space, OB odontoblasts, * subodontoblastic layer. a-e: $\times 200$; f: $\times 140$

MATERIALS AND METHODS

All experiments were performed following the guidelines of the Niigata University Intramural Animal Use and Care Committee. Five 30-day-old Wistar rats were used in this study. The animals were anesthetized by an intraperitoneal injection of chloral hydrate (400 mg/kg), and transcardially perfused with Ringer's solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The mandibles were removed and immersed in the same fixative for an additional 6 h. Following decalcification in 5% ethylenediaminetetraacetic acid (EDTA) at 4°C for three weeks, each mandible was sliced with a razor blade into four pieces (about 2 mm in thickness; see Fig. 1a-d) at right angles to the long axis of the lower incisor tooth from the apex to the incisal tip, and the pieces of tooth with surrounding bones were transversally sectioned at 40–50 μ m with a Microslicer DTK-2000 (Dosaka EM Co., Kyoto, Japan). Some specimens were cut transversely at a thickness of 20 μ m with a cryostat, thaw-mounted on poly-L-lysine-coated glass slides, and used for immunohistochemical control examinations.

For the immuno-peroxidase procedure, the sections were processed for the avidin-biotin peroxidase complex (ABC) method (HSU et al., 1981), using an anti-Hsp 25-polyclonal antibody raised in a rabbit (Stress-Gen Biotechnologies Corp., Victoria, BC, Canada), diluted 1:5000. According to the manufacturer's instructions, this antibody can recognize Hsp 25/27 from mouse, hamster, guinea pig, rat, bovine and canine cell lysates. A solution of 0.01 M phosphate-buffered saline (PBS, pH 7.4) was used to dilute the antibody and rinse the sections. The sections were then treated by two consecutive incubations with

biotinylated anti-rabbit IgG and ABC complex (Vector Lab. Inc., Burlingame, CA, USA) for 2 h each at room temperature (RT). The sites of antigen-antibody reaction were made visible by placing sections in 0.05 M Tris HCl buffer (pH 7.6) containing 0.04% 3-3'-diaminobenzidine tetrachloride and 0.002% H₂O₂. The immunostained sections were sequentially post-fixed in 1% OsO₄ reduced with 1.5% potassium ferrocyanide, dehydrated in an ascending grade of ethanol, and embedded in Epon 812 (Taab, Berkshire, UK). Semithin sections (1 μ m in thickness) were prepared and counter-stained with 0.03% methylene blue. Ultrathin sections, at a thickness of 70 nm, were double stained with uranyl acetate and lead citrate, and examined in a Hitachi H-7000 transmission electron microscope.

For confocal laser scanning microscopy, the sections were treated by consecutive incubation with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Vector Lab. Inc.), and diluted 1:100 for 1 h at RT following the incubation with the primary antibody. After washing with PBS, they were incubated with rhodamine-labeled phalloidin (Molecular Probes Inc., Eugene, OR, USA), diluted 1:100 for 1 h at RT, rinsed with PBS, and examined in a confocal laser scanning microscope (LSM 510; Carl Zeiss, Germany). The intervals between the successively scanned slices were 0.9–1.0 μ m, and one optional slice was selected for analysis of the scanned images. The line graphs in the figures (see Figs. 3c, f, i, l, 4c, f, i, l) indicate the intensity in both FITC (Hsp 25; colored green) and rhodamine (actin; colored red) across the image along a middle white line.

Immunohistochemical controls were performed by: 1) replacing the primary antibody with non-immune serum or PBS; 2) omitting the anti-rabbit IgG or the ABC complex; and 3) incubating with the primary

Fig. 3. Semithin sections (**a, d, g, j**) and confocal laser scanning microscopic images (**b, c, e, f, h, i, k, l**) of Hsp 25-IR (**a, b, d, e, g, h, j, k**) and the localization of both Hsp 25-IR and actin filaments (**c, f, i, l**) in the odontoblasts and subodontoblastic layer at different stages of dentinogenesis. **a–c.** Early stage of dentinogenesis from the specimens corresponding to Figure 1a. The subodontoblastic layer displays more intense Hsp 25-IR than the immature odontoblasts without notable cell processes. The odontoblasts show the co-localization of Hsp 25-IR and actin filaments, whereas their co-localization in the subodontoblastic layer is unclear. Both odontoblastic and subodontoblastic endothelial cells include actin filaments (*arrows*). **d–i.** Active secretory stage of dentinogenesis from the specimens corresponding to Figure 1c. In the lingual side, Hsp 25-IR is restricted to the mature odontoblasts, and intense IR is localized in both the proximal end of the cytoplasm and the bottom of cell process. The co-localization of Hsp 25-IR and actin filaments is recognizable in the cytoplasm of the odontoblasts except for their proximal and distal ends. In the labial side, the subodontoblastic layer still shows Hsp 25-IR in addition to the same co-localization of Hsp 25-IR and actin filaments in the odontoblasts. The distal terminal web of mature odontoblasts shows intense reactions for actin. **j–l.** Late stage of dentinogenesis from the specimens corresponding to Figure 1d. Near the incisal end, the odontoblast layer decreases in height, showing the co-localization of Hsp 25-IR and actin filaments. *D* dentin, *DP* dental pulp, *OR* odontoblasts, *PD* predentin, *arrowheads* capillaries, *** subodontoblastic layer. **a, d, g, j:** $\times 650$; **b, c, e, f, h, i, k, l:** $\times 400$

Fig. 3. Legend on the opposite page.

antibody preabsorbed with murine recombinant Hsp 25 (10 μ g/ml of diluted primary antibody; StressGen Biotechnologies Corp.) prior to immunocytochemistry. Further characterization of the primary antibody has been reported elsewhere (PLUMIER et al., 1997).

Since a variety of terminology has been used for odontoblasts in the process of dentinogenesis, we employ here the terms immature-, mature- and post-odontoblasts, as based on the parameters of their functional activity and nutritional supply in our previous report (OHSHIMA and YOSHIDA, 1992).

RESULTS

Immunocytochemical localization of Hsp 25

The exhibition of Hsp 25-immunoreactivity varied in the dental pulp and enamel organ according to the stages of odontogenesis (Fig. 1).

No difference in the expression of Hsp 25-immunoreaction in the odontoblasts could be found between the labial side (which was covered with enamel) and lingual side (which was covered with cementum). Intense immunoreactions for Hsp 25 were recognized in the mature odontoblasts, with highly developed cell organelles and notable processes forming a pseudostratified layer. Their immunoreactions were localized throughout their cell bodies; the most intense reactions were found both at the proximal end of the cytoplasm and the base of odontoblast cell processes within the predentin throughout dentinogenesis. However, their processes in the dentin lacked any immunoreactivity (Figs. 1b-d, 2b, c, 3d-l). In addition to immunoreactions for Hsp 25 in the immature odontoblasts without notable cell processes, the subodontoblastic layer near the apical end displayed more intense reactivity than the immature odontoblasts. These immunoreactions were uniformly found throughout the whole cytoplasm (Figs. 1a, 2a, 3a-c, 5a, c). The intense immunoreaction of Hsp 25 in the odontoblasts was exclusively localized in the cytoplasm except for such developed cell organelles

as the Golgi apparatus or rough-surfaced endoplasmic reticulum and mitochondria, i. e., in the proximal end of the cytoplasm and the bottom of cell processes. Endothelial cells, cells associating with odontoblastic capillaries, and certain cells including tubulovesicular and lysosome-like structures were negative for the immunoreaction (Fig. 5a-c). The subodontoblastic layer decreased in immunointensity for Hsp 25 with the progress of dentinogenesis except for the labial side where intense reactions for Hsp 25 remained by the incisal side of the tooth (Figs. 1, 2a-c, 3, 5d). In the dental pulp near the apical end, most of the pulp cells including fibroblasts and undifferentiated mesenchymal cells showed intense Hsp 25-immunoreactivity in the entire cytoplasm (Figs. 1a, 2a, 3a-c, 5a, c). These Hsp 25-immunopositive pulp cells decreased in number with the advance of dentinogenesis to disappear completely at the middle position of the tooth (Figs. 1c, 2b, 3d-f). The vascular components—including the endothelial and smooth muscle cells—were immunonegative for Hsp 25 in the dental pulp.

During amelogenesis, an alteration in the expression of Hsp 25-immunoreaction was recognized between the types of ameloblasts. At the early secretory stage where the enamel matrix already had been laid down, most of the ameloblasts lacked Hsp 25-immunoreactivity, in spite of weak reactions at the mesial and distal end of the cell bodies. The cellular elements of the stratum intermedium (SI) exhibited moderate reactions for Hsp 25 (Figs. 1a, 2d). At the more advanced secretory stage, Hsp 25-immunoreactions were observed throughout the cell bodies, being especially intense in the proximal end of ameloblasts. On the other hand, the cellular elements of SI became faint or negative in Hsp 25-immunoreaction (Figs. 1b, 2e, 4a-c). The expression of Hsp 25 differed considerably between the two types of ameloblasts at the mature stage: ruffle-ended ameloblasts (RA) constantly showed Hsp 25-immunoreactions which were uniformly distributed throughout the cytoplasm, whereas smooth-ended ameloblasts

Fig. 4. Semithin sections (a, d, g, j) and confocal laser scanning microscopic images (b, c, e, f, h, i, k, l) of Hsp 25-IR (a, b, d, e, g, h, j, k) and the localization of both Hsp 25-IR and actin filaments (c, f, i, l) in the ameloblasts at different stages of amelogenesis. a-f. Secretory ameloblasts (AB) and ruffle-ended ameloblasts (RA) with a ruffled border (arrows) showing Hsp 25-IR throughout the cell bodies and intense IR in the proximal end. Co-localization of Hsp 25-IR and actin filaments is observed in both the proximal and distal cytoplasm. Cells of the stratum intermedium (SI) and papillary layer cells (PC) consistently show strong reactions for actin in the cytoplasm. g-l. Smooth-ended ameloblasts (SA) and ameloblasts (AB) containing pigment vesicles showing strong Hsp 25-IR in the proximal cytoplasm and faint or no IR in the other cytoplasm. Co-localization of Hsp 25-IR and actin filaments is recognizable only in the proximal end. Papillary layer cells (PC) consistently show strong reactions for actin in the cytoplasm. E enamel or enamel space. a, d, g, j: $\times 650$; b, c, e, f, h, i, k, l: $\times 400$

Fig. 4. Legend on the opposite page.

(SA) possessed a weaker immunoreactivity at the distal end, and the other cells of the enamel organ were totally immunonegative (Figs. 1c, 2f, 4d-i). At the electron microscopic level, electron-dense thick filamentous bundles were recognized in the distal cytoplasm of both RA and SA and the junctional area between maturation ameloblasts and papillary layer cells (Fig. 6). At the later stage of enamel maturation when the cytoplasm of ameloblasts began to contain pigment vesicles, weak reactions for Hsp 25 were recognized in their cytoplasm except for intense reactions in the mesial end (Figs. 1d, 4j-l).

Confocal laser scanning microscopic observation on the co-localization of Hsp 25 and actin filaments

Confocal laser scanning microscopy clearly demonstrated the co-localization of Hsp 25 and actin filaments in both the odontoblasts and ameloblasts. During dentinogenesis, an intense immunoreaction for Hsp 25 was exclusively localized in both the proximal end of the cytoplasm and the bottom of the cell process. A moderate immunoreaction for Hsp 25 was also recognized in the other area of the odontoblasts that contained a large amount of actin. In the dental pulp, on the other hand, the distribution of the Hsp 25-immunoreaction did not correlate with the localization of actin filaments, which increased in density with the progress of dentinogenesis toward the incisal end. The distal terminal web of mature odontoblasts showed intense reactions for actin, and both odontoblastic and subodontoblastic endothelial cells included actin filaments in their cytoplasm (Fig. 3b, c, e, f, h, i, k, l).

Throughout amelogenesis, intense immunoreactions for Hsp 25 were constantly recognizable in the proximal cytoplasm of ameloblasts, where an intense reaction for actin was also observed. A similar co-localization of Hsp 25 and actin filaments was also recognized in the distal cytoplasm of both the secretory ameloblasts and RA. On the other hand, the

immunoreactivity for Hsp 25 in the distal cytoplasm became weak in the cases of both SA and the ameloblasts containing pigment vesicles at the late maturation stage, although an intense reaction for actin was recognized there. The other cellular elements of the enamel organ—SI, stellate reticulum, outer enamel epithelium, and papillary layer—consistently showed intense reactions for actin during amelogenesis (Fig. 4b, c, e, f, h, i, k, l).

The control sections after an absorption test did not show any specific immunoreactions except for the nerve bundles in the periodontium (Fig. 7).

DISCUSSION

This study clearly demonstrated a characteristic expression pattern of the immunoreaction for Hsp 25 in the odontoblasts, pulp mesenchymal cells, and ameloblasts in the rat incisor.

Dentinogenesis

The presence of immunoreactions in the odontoblasts and pulp fibroblasts as shown in this study is consistent with previous findings in the human dental pulp (SENS et al., 1996). The constant and intense Hsp 25-immunoreactions in the odontoblasts appeared to be related to the secretory activity of odontoblasts, since the rate of dentin deposition became higher from the apical to the incisal end (OHSHIMA and YOSHIDA, 1992). The odontoblasts are protein-secreting cells responsible for this active dentinogenesis, resulting in a high concentration of RNA and protein in the cytoplasm. The presence of a constitutive pool of Hsps within the odontoblasts suggests a role for Hsps as a molecular chaperone to prevent protein aggregation during and following synthesis (JAKOB et al., 1993). This idea is supported by the cytological features of the odontoblasts; the immunoreaction for Hsp 25 was exclusively localized in the cytoplasmic

Fig. 5. Electron micrographs of Hsp 25-IR in the odontoblasts and subodontoblastic layer in the lingual side at the early stage of dentinogenesis (a-c) corresponding to Figure 1a and the labial side at the middle middle portion of the tooth (d) corresponding to Figure 1c. **a.** Overview of the odontoblasts and subodontoblastic layer showing strong IR in their cytoplasm. The most intense reactions are found in both the proximal end of the cytoplasm and the bottom of the odontoblast cell processes. **b.** A higher magnification of the boxed area in **a**. The intense IR in the odontoblasts exclusively localized in the cytoplasm except for the developed cell organelles such as the Golgi apparatus, rough-surfaced endoplasmic reticulum, and mitochondria. Endothelial cells (*arrowheads*) and a cell associating with the odontoblastic capillary (*) show the absence of IR from their cytoplasm. **c.** An Hsp 25-immunonegative cell (★) containing tubulovesicular structures (*arrows*) and lysosome-like structures is observed among mesenchymal cells showing strong IR in the subodontoblastic layer. **d.** Mesenchymal cells showing strong IR remain in the subodontoblastic layer of the labial side. Note numerous Hsp 25-immunopositive fine cell processes. CL capillary lumen, OB odontoblasts, PD predentin. a: $\times 1,900$; b-d: $\times 4,600$

Fig. 5. Legend on the opposite page.

Fig. 6. Legend on the opposite page.

Fig. 7. The transverse cryosection of a control tooth incubated with the primary antibody with murine recombinant Hsp 25. This immunocontrol section does not show any specific immunoreactions except for the nerve bundles (*arrows*) in the periodontium. *AB* ameloblasts, *DP* dental pulp, *E* enamel space, *OB* odontoblasts, * periodontal ligament. $\times 40$

area without the developed cell organelles, i. e., in the proximal end of the cytoplasm and the bottom of cell processes.

Another explanation would suggest that the Hsp 25 expression in the odontoblasts can be regarded as a stress response by modulating actin dynamics and/or negative regulators of programmed cell death (ARRIGO and PRÉVILLE, 1999). From the apical to incisal end of the tooth, the odontoblasts retreat inward with the advance of dentin deposition, resulting in the formation of a pseudostratified layer by a reduction in pulp space at the middle position (OHSHIMA and YOSHIDA, 1992). This histological finding readily supposes that the odontoblasts receive mechanical stresses owing to increased cell density during the active dentin formation. This environmental change might induce the expression of intense Hsp 25-immunoreactions in these cells.

Interestingly, the localization of Hsp 25-immunoreaction in the odontoblasts resembles that of actin filaments as demonstrated in the present and previous studies (SIGAL et al., 1984; NISHIKAWA and KITAMURA, 1986, 1987; NISHIKAWA and SASA, 1989; GOLDBERG et al., 1991; WEBB et al., 1995; MOXHAM et al., 1998), although the reactions for actin in this study were weak in the mesial and distal end of the cytoplasm possessing the most intense reactions for Hsp

25. Since actin filaments have diverse roles including cell motility, the control of cell shape, and support for cell surface structures in non-muscle cells (POLLARD, 1987), those actin filaments reactive to phalloidin might play a role in resisting the pressure due to crowding in the odontoblasts (NISHIKAWA and SASA, 1989). It is reasonable to consider that Hsp 25 expression may be correlated with the formation and maintenance of the actin filaments to retain the integrity of the odontoblast layer.

It is noteworthy that immature odontoblasts, first with weak reactivity, became intensely Hsp 25-immunoreactive with the advance of the differentiation of the cells, in contrast to the dental pulp cells showing an opposite change in Hsp 25-expression. These opposing patterns of expression may be explained by the differences in cytological features between the odontoblasts and the other pulp cells. Recent studies have suggested that the accumulation of Hsp 25 in the early phase of the differentiation process occurs concomitantly with a decreased rate of cellular proliferation (MEHLEN et al., 1997). The odontoblasts are highly polarized post-mitotic cells that withdraw from the cell cycle (FRANK and NALBANDIAN, 1989). Thus, it is likely that the appearance of Hsp 25-immunoreaction in the immature odontoblasts reflects the final differentiation from the ectodermal mesen-

Fig. 6. Electron micrographs of Hsp 25-IR in the ruffle-ended ameloblasts (RA) (a-c) and smooth-ended ameloblasts (SA) (d, e) at the maturation stage corresponding to Figure 1c. **b** and **e** are higher magnifications of the boxed areas in **a** and **d**, respectively. **c**. A higher magnification of the mesial side of the RA in **a**. Electron-dense thick filamentous bundles (*arrows*) are observed in the distal cytoplasm of both RA and SA and the junctional area between maturation ameloblasts and papillary cells. Immunoreactions for Hsp 25 are diffusely recognizable in the proximal end of RA, and the other areas of RA and the SA show no clear immunoreaction at the electron microscopic level. **a**, **d**: $\times 2,600$; **b**, **c**, **e**: $\times 7,300$

chymal cells. Furthermore, cells associating with odontoblastic capillaries and certain subodontoblastic cells—including tubulovesicular and lysosome-like structures—are negative in immunoreaction for Hsp 25. These cells could be class II major histocompatibility complex (MHC)-positive cells, judging from their localization and cytological features as demonstrated in our previous study (OHSHIMA et al., 1994). Thus, Hsp 25-immunoreactions in the cells may depend on different cytological features and/or origins of the cells. This hypothesis remains to be tested in future studies to examine the expression patterns of Hsp 25-immunoreactivity among the different types of cells in the dental pulp.

Amelogenesis

A most notable finding in the active secretory stage was the localization of the Hsp 25-immunoreaction in the ameloblasts, which seems to correlate well with the secretory activity of the cells. Studies have shown that the areas with immunoreaction are localized to the terminal webs that contain actin, myosin, tropomyosin, and α -actin as major structural components (NISHIKAWA and KITAMURA, 1986; NISHIKAWA et al., 1988; GOLDBERG et al., 1991). The present confocal microscopic observation could more clearly demonstrate the co-localization of Hsp 25 and actin filaments in the secretory ameloblasts. During the enamel secretion to produce rod decussation, the secretory ameloblasts retreat backwards from the dentino-enamel junction to the enamel surface accompanied with a lateral movement (KALLENBACH, 1973; REITH and ROSS, 1973; HANAIZUMI et al., 1998). In this process, the filamentous elements of the distal terminal webs may contribute to the power-generating machinery of this lateral movement (NISHIKAWA et al., 1988, 1990; NISHIKAWA, 1992), whereas the proximal ones may serve in the formation and maintenance of intercellular sealing in the epithelial cells (OWARIBE et al., 1981; OWARIBE and MASUDA, 1982). Considering these findings together, the Hsp 25 expression observed in the cytoplasm of secretory ameloblasts may be involved in the formation and maintenance of the actin filaments by modulating actin dynamics (LAVOIE et al., 1993b; HUOT et al., 1996) to complete the integrity of the cell layer during enamel secretion. This idea is strongly supported by our preliminary examination showing that an administration of cytochalasin D, a specific and potent inhibitor of actin filament, inhibits the expression of Hsp 25-immunoreaction in the secretory ameloblasts, where actin formation is selectively damaged (authors' unpublished data).

The cellular elements of SI represented the immunoreaction for Hsp 25 only at the early secretory stage. Hsp 25-expression does not seem to correlate with mineral transport as demonstrated in previous studies (GARANT and SASAKI, 1986; SASAKI and GARANT, 1986; TAKANO et al., 1986; SASAKI et al., 1988), because its immunoreactivity disappears until the active secretory stage. It is more prudent to say that the transient Hsp 25-immunoreaction in the cells of SI is concerned with the cytodifferentiation of the immature cell types into mature ones that contain vinculin and desmoplakin, implying the presence of a junctional complex between adjacent cells (NISHIKAWA et al., 1988).

In the present study, Hsp 25-immunoreactivity was found throughout the cytoplasm of the RA, was weak in the distal cytoplasm of the SA, and not recognized in the other cells of the enamel organ. NISHIKAWA and JOSEPHSEN (1987) demonstrated cyclic changes in F-actin localization within the maturation ameloblasts: RA exhibited intense reactions for F-actin at both distal and proximal ends, whereas SA did so at their proximal ends only. The present confocal laser scanning microscopic observation demonstrated the co-localization of Hsp 25 and actin filaments in the proximal cytoplasm of ameloblasts throughout amelogenesis. In the distal cytoplasm of the maturation ameloblasts and their connective areas with papillary layer cells, furthermore, the current immunoelectron microscopic observation demonstrated the occurrence of electron-dense thick filamentous bundles, which were reported as tonofilaments in previous studies (KALLENBACH, 1968, 1970; REITH, 1970; GARANT, 1972). The present confocal microscopy showed the distribution of actin filaments in the proximal and distal terminal webs where the thick tonofilaments were localized. Thus, actin filaments could be co-localized with the tonofilaments in the maturation ameloblasts.

In spite of the consistent presence of actin filaments, Hsp 25-immunoreactions in the distal cytoplasm of maturation ameloblasts vary depending on the presence of the ruffled border. Hsp 25 is known to correlate with actin dynamics (LAVOIE et al., 1993b; HUOT et al., 1996); the administration of cytochalasin D induces a complete disappearance of the ruffled border-clear zone complexes in osteoclasts (SASAKI et al., 1993). As described above, an administration of cytochalasin D depresses Hsp 25-immunoreaction in the ameloblasts at the maturation as well as secretory stages (authors' unpublished data). Taken together, these findings strongly suggest that Hsp 25-immunoreaction might be concerned with the formation and maintenance of the ruffled border of RA.

This idea is supported by the present findings that, at the pigmentation stage, the distal cytoplasm of the ameloblasts without a ruffled border became negative in Hsp 25-immunoreaction. However, we cannot deny the relationship between the Hsp 25-expression in the RA and the high Ca^{2+} incorporation in the enamel of the apical two thirds of each RA region (TAKANO et al., 1982; KAWAMOTO and SHIMIZU, 1990), since Ca^{2+} and phospholipid-binding proteins called annexins have been shown to co-localize with actin in the secretory ameloblasts and odontoblasts of the rat incisor (GOLDBERG et al., 1991). As for their operation in energy-requiring events in the maturing enamel, these ameloblasts form a functional unit with the papillary layer cells (OHSHIMA et al., 1998). It is conceivable that the Hsp 25-immunoreaction in the enamel organ does not correlate with this process, because the papillary layer cells exhibited no immunoreaction in the present study.

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REFERENCES

- ARRIGO, A.-P. and X. PRÉVILLE: Role of Hsp 25 and related proteins. In: (ed. by) D. S. LATCHMAN: Handbook of experimental pharmacology, Vol. 136. Stress proteins, Chap. 5. Springer, Berlin-New York, 1999 (p. 101-132).
- CIOCCA, D. R., S. OESTERREICH, G. C. CHAMNESS, W. L. MCGUIRE and S. A. W. FUQUA: Biological and clinical implications of heat shock protein 25,000 (Hsp 25): a review. *J. Nat. Cancer Inst.* 85: 1558-1570 (1993).
- FRANK, R. M. and J. NALBANDIAN: Development of dentine and pulp. In: (ed. by) A. OKSCHE and L. VOLL-RATH: Handbook of microscopic anatomy, Vol. 5, Part 6. Teeth. Springer, Berlin-Heidelberg-New York, 1989 (p. 73-171).
- GARANT, P. R.: The demonstration of complex gap junctions between the cells of the enamel organ with lanthanum nitrate. *J. Ultrastr. Res.* 40: 333-348 (1972).
- GARANT, P. R. and T. SASAKI: Ultracytochemistry of ouabain-sensitive K^{+} -dependent p-nitrophenyl phosphatase in rat incisor enamel organ. *Anat. Rec.* 216: 1-9 (1986).
- GOLDBERG, M., J. FEINBERG, S. LECOLLE, M. A. KAETZEL, D. RAINTEAU, J. L. LESSARD, J. R. DEDMAN and S. WEINMAN: Co-distribution of annexin VI and actin in secretory ameloblasts and odontoblasts of rat incisor. *Cell Tiss. Res.* 263: 81-89 (1991).
- HANAIZUMI, Y., Y. KAWANO., H. OHSHIMA, M. HOSHINO, K. TAKEUCHI and T. MAEDA: Three-dimensional direction and interrelationship of prisms in cuspal and cervical enamel of dog tooth. *Anat. Rec.* 252: 355-368 (1998).
- HANASH, S. M., J. R. STRAHLER, Y. CHAN, R. KUICK, D. TEICHROEW, J. V. NEEL, N. HAILAT, D. R. KEIM, J. GRATIOT-DEANS, D. UNGAR, R. MELHEM, X. X. ZHU, P. ANDREW, F. LOOTSPEICH, C. ECKERSKORN, E. CHU, I. ALI, D. A. FOX and B. C. RICHARDSON: Data base analysis of protein expression patterns during T-cell ontogeny and activation. *Proc. Nat. Acad. Sci. USA* 90: 3314-3318 (1993).
- HSU, S. M., L. RAINE and H. FANGER: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29: 577-580 (1981).
- HUOT, J., G. ROY, H. LAMBERT, P. CHRÉTIEN and J. LANDRY: Increased survival after treatments with anticancer agents of Chinese hamster cells expressing the human Mr 25,000 heat shock protein. *Cancer Res.* 51: 5245-5252 (1991).
- HUOT, J., F. HOULE, D. R. SPITZ and J. LANDRY: HSP25 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. *Cancer Res.* 56: 253-259 (1996).
- JAKOB, U., M. GAESTEL, K. ENGEL and J. BUCHNER: Small heat shock proteins are molecular chaperones. *J. Biol. Chem.* 268: 1517-1520 (1993).
- KALLENBACH, E.: Fine structure of rat incisor ameloblasts during enamel maturation. *J. Ultrastr. Res.* 22: 90-119 (1968).
- : Fine structure of rat incisor enamel organ during late pigmentation and regression stages. *J. Ultrastr. Res.* 30: 38-63 (1970).
- : The fine structure of Tomes' process of rat incisor ameloblasts and its relationship to the elaboration of enamel. *Tiss. Cell* 5: 501-524 (1973).
- KAWAMOTO, T. and M. SHIMIZU: Changes in the mode of calcium and phosphate transport during rat incisal enamel formation. *Calcif. Tiss. Int.* 46: 406-414 (1990).
- LANDRY, J., P. CHRÉTIEN, H. LAMBERT, E. HICKEY and L. A. WEBER: Heat shock resistance conferred by expression of the human HSP 25 gene in rodent cells. *J. Cell Biol.* 109: 7-15 (1989).
- LAVOIE, J. N., G. GINGRAS-BRETON, R. M. TANGUAY and J. LANDRY: Induction of Chinese hamster HSP 25 gene expression in mouse cells confers resistance to heat shock. HSP 25 stabilization of the microfilament organization. *J. Biol. Chem.* 268: 3420-3429 (1993a).
- LAVOIE, J. N., HICKEY, E., WEBER, L. A. and LANDRY, J.: Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 25. *J. Biol. Chem.* 268: 24210-24214 (1993b).
- MEHLEN, P., X. PREVILLE, P. CHAREYRON, J. BRIOLAY, R. KLEMENZ and A.-P. ARRIGO: Constitutive expression of human hsp25, *Drosophila* hsp25, or human alpha B-crystallin confers resistance to TNF- and oxidative

- stress-induced cytotoxicity in stably transfected murine L929 fibroblasts. *J. Immunol.* 154: 363-374 (1995).
- MEHLEN, P., C. KRETZ-REMY, X. PREVIL and A.-P. ARRIGO:** Human hsp25, Drosophila hsp25 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFalpha-induced cell death. *EMBO J.* 15: 2695-2706 (1996a).
- MEHLEN, P., K. SCHULZE-OSTHOFF and A.-P. ARRIGO:** Small stress proteins as novel regulators of apoptosis. Heat shock protein 25 blocks Fas/APO-1- and staurosporine-induced cell death. *J. Biol. Chem.* 251: 16510-16514 (1996b).
- MEHLEN, P., A. A. MEHLEN, J. GODET and A.-P. ARRIGO:** Hsp25 as a switch between differentiation and apoptosis in murine embryonic stem cells. *J. Biol. Chem.* 252: 31657-31665 (1997).
- MOXHAM, B. J., P. P. WEBB, M. BENJAMIN and J. R. RALPHS:** Changes in the cytoskeleton of cells within the periodontal ligament and dental pulp of the rat first molar tooth during ageing. *Eur. J. Oral Sci.* 106 (Suppl.): 376-383 (1998).
- NISHIKAWA, S.:** Correlation of the arrangement pattern of enamel rods and secretory ameloblasts in pig and monkey teeth: a possible role of the terminal webs in ameloblast movement during secretion. *Anat. Rec.* 232: 466-478 (1992).
- NISHIKAWA, S. and K. JOSEPHSEN:** Cyclic localization of actin and its relationship to junctional complexes in maturation ameloblasts of the rat incisor. *Anat. Rec.* 219: 21-31 (1987).
- NISHIKAWA, S. and H. KITAMURA:** Localization of actin during differentiation of the ameloblast, its related epithelial cells and odontoblasts in the rat incisor using NBD-phalloidin. *Differentiation* 30: 237-243 (1986).
- : Microtubules, intermediate filaments, and actin filaments in the odontoblast of rat incisor. *Anat. Rec.* 219: 144-151 (1987).
- NISHIKAWA, S. and S. SASA:** Immunofluorescent localization of myosin, alpha-actinin and tropomyosin in odontoblast microfilament bundles of the rat incisor. *Cell Struct. Funct.* 14: 407-414 (1989).
- NISHIKAWA, S., K. FUJIWARA and H. KITAMURA:** Formation of the tooth enamel rod pattern and the cytoskeletal organization in secretory ameloblasts of the rat incisor. *Eur. J. Cell Biol.* 47: 222-232 (1988).
- NISHIKAWA, S., S. TSUKITA, S. TSUKITA and S. SASA:** Localization of adherens junction proteins along the possible sliding interface between secretory ameloblasts of the rat incisor. *Cell Struct. Funct.* 15: 245-249 (1990).
- OHSHIMA, H. and S. YOSHIDA:** The relationship between odontoblasts and pulp capillaries in the process of enamel- and cementum-related dentin formation in rat incisors. *Cell Tiss. Res.* 268: 51-63 (1992).
- OHSHIMA, H., I. KAWAHARA, T. MAEDA and Y. TAKANO:** The relationship between odontoblasts and immunocompetent cells during dentinogenesis in rat incisors: an immunohistochemical study using OX6-monoclonal antibody. *Arch. Histol. Cytol.* 57: 435-447 (1994).
- OHSHIMA, H., T. MAEDA and Y. TAKANO:** Cytochrome oxidase activity in the enamel organ during amelogenesis in rat incisors. *Anat. Rec.* 252: 519-531 (1998).
- OWARIBE, K. and H. MASUDA:** Isolation and characterization of circumferential microfilament bundles from retinal pigmented epithelial cells. *J. Cell Biol.* 95: 310-315 (1982).
- OWARIBE, K., R. KODAMA and G. EGUCHI:** Demonstration of contractility of circumferential actin bundles and its morphogenetic significance in pigmented epithelium in vitro and in vivo. *J. Cell Biol.* 90: 507-514 (1981).
- PLUMIER, J. C., D. A. HOPKINS, H. A. ROBERTSON and R. W. CURRIE:** Constitutive expression of the 25-kDa heat shock protein (Hsp 25) in sensory and motor neurons of the rat nervous system. *J. Comp. Neurol.* 384: 409-428 (1997).
- POLLARD, T. D.:** Actin. *Curr. Opin. Cell Biol.* 2: 33-40 (1990).
- REITH, E. J.:** The stages of amelogenesis as observed in molar teeth of young rats. *J. Ultrastr. Res.* 30: 111-151 (1970).
- REITH, E. J. and M. H. ROSS:** Morphological evidence for the presence of contractile elements in secretory ameloblasts of the rat. *Arch. Oral Biol.* 18: 445-448 (1973).
- SAMALI, A. and T. G. COTTER:** Heat shock proteins increase resistance to apoptosis. *Exp. Cell Res.* 223: 163-170 (1996).
- SASAKI, T. and P. R. GARANT:** Ultracytochemical demonstration of ATP-dependent calcium pump in ameloblasts of rat incisor enamel organ. *Calcif. Tiss. Int.* 39: 86-96 (1986).
- SASAKI, T., K. TADOKORO, T. YANAGISAWA, S. HIGASHI and P. R. GARANT:** H⁺-K⁺-ATPase activity in the rat incisor enamel organ during enamel formation. *Anat. Rec.* 221: 823-833 (1988).
- SASAKI, T., K. DEBARI and N. UDAGAWA:** Cytochalasin D reduces osteoclastic bone resorption by inhibiting development of ruffled border-clear zone complex. *Calcif. Tiss. Int.* 53: 217-221 (1993).
- SENS, D. A., J. P. MCGUIRT, W. KHAN, J. H. TODD and R. M. HOWELL:** Expression of heat shock protein 25 in adult human third molar dental pulp. *J. Oral Pathol. Med.* 25: 382-387 (1996).
- SHAKOORI, A. R., A. M. OBERDORF, T. A. OWEN, L. A. WEBER, E. HICKEY, J. L. STEIN, J. B. LIAN and G. S. STEIN:** Expression of heat shock genes during differentiation of mammalian osteoblasts and promyelocytic leukemia cells. *J. Cell Biochem.* 48: 257-287 (1992).
- SIGAL, M. J., J. E. AUBIN, A. R. TEN CATE and S. PITARU:** The odontoblast process extends to the dentinoenamel junction: an immunocytochemical study of rat dentine. *J. Histochem. Cytochem.* 32: 872-877 (1984).
- SPECTOR, N. L., W. SAMSON, C. RYAN, J. GRIBBEN, W. URBA, W. J. WELCH and L. M. NADLER:** Growth arrest of human B lymphocytes is accompanied by induction of the low molecular weight mammalian heat shock protein (Hsp 28). *J. Immunol.* 148: 1668-1673 (1992).

- SPECTOR, N. L., C. RYAN, W. SAMSON, H. LEVINE, L. M. NADLER and A.-P. ARRIGO:** Heat shock protein is a unique marker of growth arrest during macrophage differentiation of HL-60 cells. *J. Cell Physiol.* 156: 619-625 (1993).
- STAHL, J., A. M. WOBUS, S. IHRIG, G. LUTSCH and H. BIELKA:** The small heat shock protein hsp25 is accumulated in P19 embryonal carcinoma cells and embryonic stem cells of line BLC6 during differentiation. *Differentiation* 51: 33-37 (1992).
- TAKANO, Y., M. A. CRENSHAW, J. W. BAWDEN, L. HAMMARSTROM and S. LINDSKOG:** The visualization of the patterns of ameloblast modulation by the glyoxal bis (2-hydroxyanil) staining method. *J. Dent. Res.* 61 (special issue): 1580-1587 (1982).
- TAKANO, Y., H. OZAWA and M. A. CRENSHAW:** Ca-ATPase and ALPase activities at the initial calcification sites of dentin and enamel in the rat incisor. *Cell Tiss. Res.* 243: 91-99 (1986).
- WEBB, P. P., B. J. MOXHAM, J. R. RALPHS and M. BENJAMIN:** Cytoskeleton of the mesenchymal cells of the rat dental papilla and dental pulp. *Connect. Tiss. Res.* 32: 51-76 (1995).
- WU, W. and M. J. WELSH:** Expression of the 25-kDa heat-shock protein (HSP 25) correlates with resistance to the toxicity of cadmium chloride, mercuric chloride, cis-platinum (II)-diammine dichloride, or sodium arsenite in mouse embryonic stem cells transfected with sense or antisense HSP 25 cDNA. *Toxicol. Appl. Pharmacol.* 141: 330-339 (1996).

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