Pulpal responses to cavity preparation in aged rat molars

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Abstract The dentin-pulp complex is capable of repair after tooth injuries including dental procedures. However, there are few available data concerning aged changes in pulpal reactions to such injuries. The present study aimed to clarify the capability of defense of aged pulp by investigating the responses of odontoblasts and class II major histocompatibility complex (MHC)-positive cells to cavity preparation in aged rat molars (300-360 d) and comparing the results with those in young adult rats (100 d). In untreated control teeth, intense heat-shock protein (HSP)-25- and nestin-immunoreactivity (IR) was found in the odontoblasts, whereas class II MHC-positive cells were densely distributed in the periphery of the pulp. Cavity preparation caused the two types of pulpal reactions showing the different extent of damage in the aged rats. In the case of severe damage, the destruction of the odontoblast layer was conspicuous at the affected site. Twelve h after cavity preparation, numerous class II MHC-positive cells appeared along the pulp-dentin border, but subsequently disappeared together with HSP-25-immunopositive cells, and finally newly differentiated odontoblast-like cells took the place of the degenerated odontoblasts and acquired HSP-25- and nestin-IR by postoperative 3 d. In the case of mild damage, there seemed no remarkable changes in the odontoblasts after operation, and some could survive through the experimental stages. These findings indicate that aged pulp tissue still possesses the defense capacity, and that a variety of reactions could occur depending on the difference in the status of dentinal tubules and/or odontoblast processes in individuals.

Key words dental cavity preparation; heat-shock proteins; histocompatibility antigens class II; odontoblasts; rats (Wistar).

Introduction

A unique feature of dentin is that it is a mineralized tissue that surrounds the pulp, an unmineralized tissue. Dental pulp not only functions to provide nutritional and sensory properties to dentin, but also has its own reparative capacity (Goldberg and Smith 2004). Thus, the dentin-pulp complex is capable of repair after tooth injuries such as caries, attrition, abrasion, and dental procedures including cavity preparation. According to the aging of the individuals, the dentin-pulp complex undergoes age-related changes, resulting in a decreasing volume of the pulp chamber and root canal, decrease in cell density, reduction in sensitivity, occurrence of dystrophic calcification, gradual reduction in the dentinal tubule diameter, retraction of the odontoblast processes, and so on (Morse 1991; Fried 1992; Murray et al. 2002; Lovschall et al. 2002; Nanci 2003). These age-related changes may influence the competent pulp tissue capable of repair following tooth injuries. An understanding of the exact biological properties of aged pulp is valuable for the proper dental treatment of aged teeth. However, there are few available data concerning aged changes in pulpal reactions to tooth injuries.

The procedure of cavity preparation induces destructive changes in odontoblasts at the affected site as well as an acute inflammatory reaction (Ohshima 1990). If the odontoblasts survive, they are capable of depositing further reactionary dentin. If not, pulpal mesenchymal cells take the place of the degenerated odontoblasts to differentiate into odontoblast-like cells resulting in the formation of reparative dentin (Smith 2002). This phenomenon may indicate that dental pulp stem cells (Gronthos et al. 2002) exist in the pulp tissue. A recent study has provided evidence that the pulpal defense reaction to cavity preparation in aged rats did not differ

markedly from that in young adult rats (Izumi et al. 2002). In such study, an accumulation of immunocompetent cells occurred along the pulp-dentin border in aged rats as well as young adult rats. The most abundant immunocompetent cells in the dental pulp are those cells with the class II major histocompatibility complex (MHC) antigen (Jontell et al. 1987, 1988, 1991, 1998; Jontell and Bergenholtz 1992; Okiji et al. 1992; Ohshima et al. 1994). These class II MHC-positive cells show characteristic reaction patterns under various experimental conditions such as tooth grinding (Ohshima et al. 1995, 2003), tooth replantation (Rungvechvuttivittaya et al. 1998, Shimizu et al. 2000; Nakakura-Ohshima et al. 2003) and carious teeth (Yoshiba et al. 1996; Izumi et al. 1996; Kamal et al. 1997; Sakurai et al. 1999). However, how tooth injury affects the odontoblasts and the regenerative properties of the pulp tissue remains to be elucidated in aged pulp tissue.

Heat-shock protein (HSP)-25 is a small molecular protein that belongs to a family of small HSPs and is a homolog of human HSP-27 (HSP-25 is applied to its homologue in this paper) (Arrigo and Préville 1999). HSP-25 synthesis is transiently induced in cells in the response to the many physiological and environmental stresses they encounter. HSP-25 functions as a molecular chaperone (Jakob et al. 1993), a suppresser of apoptosis (Mehlen et al., 1996, 1997), an inhibitor of actin polymerization (Miron et al., 1991), and a modulator of actin dynamics (Lavoie et al., 1993; Huot et al., 1996). In addition to stressful and normal conditions, the transient expression of HSP-25 has been reported in various cells during development and cell differentiation (Arrigo and Préville 1999). The odontoblasts also show a stage-specific expression pattern of HSP-25-immunoreactivity (IR) in intact teeth (Ohshima et al. 2000, 2002) and under experimental conditions (Ohshima et al. 2001a, 2001b, 2003;

Nakakura-Ohshima et al. 2003; Suzuki et al. 2004; Tate et al. 2006), suggesting that this protein is a useful marker for the differentiation of odontoblasts during the pulpal healing process. This notion is supported by the our recent study that 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 (Nakasone et al. 2006). On the other hand, the intermediate filament nestin could be used as a specific marker for the odontoblasts (Terling et al. 1995), because nestin is exclusively expressed in matured odontoblasts in the erupted tooth and its surrounding tissue. Furthermore, odontoblast processes also show nestin-IR in addition to their cell bodies (About et al 2000) and it is conceivable that there is no unique phenotypic marker of secretory odontoblasts (Goldberg and Smith, 2004). Thus, the combination of plural cellular makers such as HSP-25 and nestin may be valuable for monitoring the degeneration/regeneration of odontoblasts during healing process after tooth injury.

To date, the responses of immunocompetent cells to cavity preparation in aged pulp have been reported to be almost identical to those in young adult pulp (Izumi et al. 2002). However, the cellular events in the pulpal healing process following cavity preparation in aged rats remain to be fully understood. This study was therefore undertaken to examine the initial responses of odontoblasts and class II MHC-positive cells to cavity preparation in aged rat molars by immunocytochemistry for HSP-25, nestin, and the class II MHC antigen.

Materials and methods

Procedure of cavity preparation

All experiments were performed following the Guidelines of the Niigata University Intramural Animal Use and Care Committee. Thirty six Wistar rats, 100 and 300-360 d old, were used in this study. Under anesthesia by an intraperitoneal injection of chloral hydrate (350 mg/kg), a groove-shaped cavity (the thickness of the remaining dentin being around 150-200 μm) was prepared on the medial surface of the upper-left first molar by an air turbine with a tungsten carbide bur (diameter of 0.6 mm) under water-cooling after gingivectomy. The cavity received no further treatment such as air drying, etching, or filling. The upper-left first molar of the same animal was used as a control. The region selected for observation was the periphery of the pulp tissue in the mesial coronal portion beneath the dentinal tubules that would suffer damage (Fig 3d).

Histological procedure

Materials were collected in groups of five animals at intervals of 0, 6, 12 and 24 h, and 3, and 5 d from 300-360 d rats and three animals at intervals of 12 h, and 3 d from 100 d rats after cavity preparation. At each stage, the animals were anesthetized by an intraperitoneal injection of chloral hydrate (350 mg/kg) and transcardially perfused with physiological saline followed with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). The maxillae including both the prepared and control teeth were removed *en bloc* and immersed in the same fixative for an additional 12 h. Following decalcification in a 4.13% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution for 6 wk at 4°C, the samples were processed for either embedding in paraffin or cryosection. The tissue blocks for cryosection were equilibrated in a 30% sucrose

solution for cryoprotection. The specimens were cut sagittally at a thickness of 25-50 μm with a freezing microtome (FX-801: Yamato Kohki Co. Ltd., Tokyo, Japan), collected into cold phosphate-buffered saline (PBS), and treated as free-floating sections. The paraffin-embedded teeth were cut sagittally at about 4 μm. The paraffin sections were mounted on MAS-coated glass slides and stained with Goodpasture Gram stain in addition to immunohistochemical analysis. Throughout the experimental stages, there were no Gram-positive bacteria in the dental pulp, although Gram-positive cocci were recognized in the prepared cavity (Fig 1).

Immunohistochemical analysis

For the immuno-peroxidase procedure, frozen sections were processed for the avidin-biotin peroxidase complex (ABC) method using either an anti-HSP-25 polyclonal (diluted 1:5000; StressGen Biotechnologies Corp., Victoria, BC, Canada), an anti-nestin monoclonal (diluted to 1:2000; Chemicon International Inc, Temecula, CA, USA), or an OX6 monoclonal (diluted 1:2000; Serotec Ltd., Oxford, UK) antibody. Our protocol for HSP-25- and OX6-immunohistochemistry has been shown in previous reports (Ohshima et al. 2000; Shimizu et al. 2000). The immunostained sections were thaw-mounted onto silane-coated glass slides, and stained with 0.03% methylene blue. The paraffin sections were processed for nestin-immunohistochemisry, and our immunohistochemical protocol was basically the same as the above protocol.

For immunocytochemistry for HSP-25 at the electron microscopic level, the immunostaining procedure was the same as described above, except for the inhibition of endogeneous peroxidase. The immunostained sections were subsequently postfixed in 1% OsO_4 reduced with 1.5% potassium ferrocyanide, dehydrated in an ascending series

of ethanol, and finally embedded in Epon 812 (Taab, Berkshire, UK). Plastic sections (1 μm in thickness) were stained with 0.03% methylene blue. Ultrathin sections (70 nm in thickness) were doubly stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 transmission electron microscope.

Immunohistochemical controls were performed by: 1) replacing the primary antibody with non-immune serum or PBS; and 2) omitting the anti-rabbit IgG, the anti-mouse IgG or the ABC complex. These immunostained sections did not contain any specific immunoreactions.

Statistical analysis of OX6-IR

We compared the OX6-IR at the periphery of the aged pulp with that of the young adult pulp (control, 12 h, and 3 d groups). The immunostained sections with 25 μm in thickness were used for statistical analysis. Quantitative analysis was performed in 2-4 sections for each group. We selected the grid (300 x 150 μm^2) including the predentin and the odontoblast and subodontoblastic layer (Figs 2b, d, f, 3e, 4f, j), and the immunoreactive area fractions (%) of each specimen were calculated using image-analysis software (WinRoof: Mitani Corp Ltd, Fukui, Japan). All data were presented as the means and standard deviations (SD) of each group. The comparisons among two groups (aged and young adult pulp) were performed by Student's *t*-test.

Results

Pulpal responses in young adult rats

In the control, HSP-25-immunohistochemistry in the dental pulp demonstrated intense IR in the odontoblasts, but a weak one in other cellular elements including nerve fibers and blood vessels (Fig 2a). Many OX6-immunopositive cells were widely distributed throughout the dental pulp of the rat molars, predominantly at the periphery of the pulp tissue. Most of the subodontoblastic immunopositive cells exhibited dendritic profiles, and some of them extended their processes into the odontoblast layer (Fig 2b). Twelve h after cavity preparation, the pulp-dentin border completely lost the IR for HSP-25, in spite of the occurrence of some cells there. HSP-25-immunopositive cells were discernible beneath the degenerated odontoblasts (Fig 2c). In contrast, OX6-immunopositive cells accumulated at the affected area along the pulp-dentin border; they frequently extended their processes into the dentinal tubules (Fig 2d). Three d after operation, HSP-25-immunoreactive plump cells, newly differentiated odontoblast-like cells, lined up in the proper odontoblast layer (Fig 2e). OX6-immunopositive cells with dendritic profiles were exclusively located beneath the odontoblast-like cells (Fig 2f).

Controls in aged rats

HSP-25-IR in the dental pulp was almost identical with that in the young adult rats except for the weak positive cells in the pulp floor. The progressive attrition, tertiary dentin formation at the enamel-free areas, and considerable cementum deposition were easily recognized as age-related changes, resulting in the shallow occlusal grooves (Fig 3a, c). The immunoreaction within odontoblasts was observed in the cell bodies and

cell processes limited within the predentin (Fig 3e). Many OX6-immunopositive cells were widely distributed throughout the dental pulp, predominantly at the periphery of the pulp tissue (Fig 3b). Most of the subodontoblastic immunopositive cells displayed dendritic profiles, and the pulpo-dentinal border zone including the odontoblast cell layer and predentin contained OX6-positive cells in the untreated control teeth (Fig 3d). OX6-immunopositive cells in the periphery were considerably decreased in number in the root region (Fig 3f). Intense nestin-IR was observed in the all odontoblasts and their cellular processes, and the odontoblast layer at the pulp floor lacked continuity in addition to losing the subodontoblastic cell-rich zone (Fig 3g, h).

Immediately after cavity preparation in aged rats

Cavity preparation caused the two types of pulpal reactions showing the different extent of damage in the early stages: severe (Figs 4a-j, 5d, f, h) and mild (Fig 5a-c, e, g). In the case of mild damage, the odontoblast layer showed no changes in the IR for HSP-25 through all the experimental stages (Fig 5a), whereas OX6-positive cells temporarily aggregated along the pulp-dentin border at 12-24 h (Fig 5b). In the case of severe damage, the odontoblasts under the prepared cavity suffered severe damage; many odontoblasts at the affected site lost HSP-25-immunoreaction (Fig 4a, c). However, HSP-25-positive cells intermingled in the damaged odontoblast cell layer (Fig 4c). The OX6-immunopositive cells showing a dendritic appearance at the affected site shifted inwards together with the damaged odontoblasts (Fig 4b, d).

Six h after cavity preparation in aged rats

In the case of severe damage, the damaged odontoblasts with HSP-25-IR showed a round profile in the impaired odontoblast layer (data not shown). In the case of mild damage, the odontoblast layer maintained cell continuity at the light microscopic level (Fig 5e), but some odontoblasts suffered degeneration and presumably dendritic cells with tubulovesicular structures and polymorphonuclear leukocytes migrated into the exposed dentinal tubules at the electron microscopic level (Fig 5g).

Twelve h after cavity preparation in aged rats

In the case of severe damage, the damaged odontoblast layer appeared to be almost the same as those in the previous stage (Fig 4e). In contrast, the OX6-immunopositive cells accumulated at the affected area along the pulp-dentin border and frequently extended their processes deep into the dentinal tubules (Figs 4f). The electron microcopy demonstrated that presumably dendritic cells with tubulovesicular structures and polymorphonuclear leukocytes accumulated at the pulp-dentin border, and that odontoblasts that suffered damage showing a round appearance maintained their IR for HSP-25 (Fig 5f, h). In the case of mild damage, on the other hand, the odontoblast looked quite normal in the IR for HSP-25 (Fig 5a), whereas numerous OX6-immunopositive cells aggregated at the pulp-dentin border and extended their cellular processes into the dentinal tubules (Fig 5b). Nestin-IR clearly demonstrated the distinction between severe (Fig 5d) and mild (Fig 5c) damage; the odontoblasts that suffered damage lost their IR for nestin in both the cell bodies and cellular processes in the case of severe damage.

Twenty-four h after cavity preparation in aged rats

No apparent immunoreaction for HSP-25 persisted at the pulp-dentin border, whereas HSP-25-IR remained in the subodontoblastic layer apart from the predentin (Fig 4g). OX6-immunopositive cells remained along the pulp-dentin border, but were reduced in number from the previous stage (Fig 4h).

Three to 5 d after cavity preparation in aged rats

After 3-5 d, the HSP-25-immunoreactive odontoblast-like cells lined up in the proper odontoblast layer, while the OX6-immunopositive cells were located beneath the newly differentiated odontoblast-like cells (Fig 4i, j).

Statistical analysis of OX6-IR

Statistical analysis of OX6-IR showed that the fractions of the immunoreactive area at the periphery of the pulp tissue in aged molars were significantly larger than those in young adult molars in the control and 3 d after cavity preparation (Fig 6).

Discussion

The present immunocytochemical study clearly demonstrated the pulpal reaction to cavity preparation in aged rat molars by using the antibodies to HSP-25, nestin, and class II MHC antigen, and that cavity preparation caused the two types of pulpal reactions showing the different extent of damage. In the case of severe damage, most odontoblasts, otherwise probably all, in the area that suffered damage were degenerated, newly differentiated odontoblast-like cells arranged at the pulp-dentin border judging from the results in IR for HSP-25 and nestin, and class II MHC-positive cells temporarily appeared at the pulp-dentin border during the pulpal healing process following cavity preparation. These chronological changes in both the odontoblast-lineage and class II MHC-positive cells in aged rats in this study is consistent with those in young adult rats in the present and previous studies (Ohshima et al. 2003; Nakakura-Ohshima et al. 2003). Our previous experimental studies have repeatedly provided evidence that the appearance of class II MHC-positive dendritic cells at the pulp-dentin border is necessary for proper pulpal healing after tooth injuries such as cavity preparation and tooth replantation, suggesting the possibility that these dendritic cells play an regulatory role in the differentiation of odontoblast-lineage cells (Shimizu et al. 2000; Ohshima et al. 2003; Nakakura-Ohshima et al. 2003; Suzuki et al., 2004; Tsukamoto-Tanaka et al. 2006) after the damaged odontoblasts degenerate immediately after surgery (Ohshima 1990; Bronckers et al. 1996; Kitamura et al. 2001) and are phagocytized by class II MHC-positive and negative macrophages (Ohshima et al. 2003). Another experimental study using aged molar teeth also reported the appearance of immunocompetent cells including class II MHC-positive cells at 1 d after cavity preparation (Izumi et al. 2002). Furthermore, our electron microscopic

observation confirmed that presumably dendritic cells with a tubulovesicular structure, which is a characteristic cell organelle in the dendritic cells (Ohshima et al. 1994, 1999), appeared at the pulp-dentin border at 6-12 h after tooth drilling. Thus, the regenerative properties of the dentin-pulp complex and the cellular responses of immunocompetent cells including dendritic cells after tooth injury are maintained even in aged dentin-pulp complex.

Our most noteworthy finding is that the odontoblast layer showed no changes in the IR for HSP-25 through all experimental stages in the case of mild damage, but that OX6-positive cells temporarily aggregated along the pulp-dentin border at 12-24 h as well as those in the case of severe damage. This difference in tissue reaction between severe and mild damage may be explained by the differences in the status of dentinal tubules and/or odontoblast processes depending on the aged individual. The issue of the exact length of odontoblastic cell processes in the dentinal tubules is still controversial (Holland 1990; Ten Cate 1998). Some researchers have claimed that they extend to the dentino-enamel junction or peripheral dentin (Gunji and Kobayashi 1983; Sigal et al. 1985; Frank and Steuer, 1988), whereas others demonstrated that they are limited to the inner pulpal third of the dentin (Thomas and Carella 1984; Byers and Sugaya 1995; Yoshiba et al., 2002). Whether it is true or not, the extent of odontoblast processes differs depending on the location of the dentin, the age of the teeth (Tsuchiya et al. 2002), and the species. Under the current experimental model, the drilling of teeth essentially influenced the dentinal tubules and/or the odontoblast processes clearly demonstrated by the loss of HSP-25-IR in the Figure 4a, and that the thickness of the remaining dentin being around 200 μm totally destroyed all affected odontoblasts in the young adult teeth (Ohshima 1990; Ohshima et al. 2001b, 2003). However, a variety of

reactions could occur even under the same experimental model in aged teeth. Furthermore, mild responses (odontoblasts retain HSP-25-IR) also occur at the shallow edges of severe injuries, indicating that odontoblast processes may not extend to the dentino-enamel junction. Thus, it is reasonable that the differences in the status of dentinal tubules and/or odontoblast processes in individuals could induce a variety of damage in the dental pulp. Interestingly, even in the case when the odontoblast layer shows continuous HSP-25-immunoreaction at the light microscopic level, some odontoblasts are degenerated following cavity preparation, and inflammatory reactions occur in the pulp-dentin border at the cellular level. Individual odontoblasts may suffer a variety of damage depending on the length of their cellular processes (Fig. 7).

The current results confirmed the age-related changes in the dental pulp: progressive attrition, reparative dentin formation at the enamel-free areas, and considerable cementum deposition, resulting in shallow occlusal grooves. Previous studies reported that the density of class II MHC-positive cells was maintained at a relatively high level in aged rats (Okiji et al. 1996; Izumi et al., 2002). The results are basically the same as the findings of the present study where the fractions of the class II MHC-immunoreactive area in aged molars were significantly larger than those in young adult molars in the control and 3 d after operation, although how to take the image data from the sections for quantitative analysis was different between the present and previous studies. Furthermore, the existence of class II MHC-positive cells in the predentin reminds us of human dendritic cells in the matured dental pulp (Ohshima et al. 1999). Further study is needed to clarify whether or not the dendritic cells make contact with the several odontoblast processes as they do in human dental pulp, because

these cells are supposed to have certain regulatory functions on the odontoblasts under physiological conditions (Ohshima et al. 1999).

Another notable age-related change is that the odontoblast layer lost its continuity in addition to losing the subodontoblastic cell-rich zone in the pulp floor. Since the odontoblast-lineage cells are supposed to reside in the subodontoblastic layer (Ruch 1995), the loss of odontoblasts is attributed to the lack of a progenitor cell pool. The evidence that coronal odontoblasts maintain cell continuity and class II MHC-positive cells are densely distributed at the periphery of coronal pulp tissue is attracting interest in the defense mechanism in dental pulp, indicating that the portion susceptible to exogenous challenges is equipped with regenerative capability and the competency to recruit dendritic cells at the pulp-dentin border after tooth injury. In conclusion, aged pulp tissue still possesses the same rapid rate of recovery as that in the young adult teeth, and the current results will provide fundamental knowledge for the proper dental treatment of aged teeth.

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References

About I, Laurent-Maquin D, Lendahl U, Mitsiadis TA (2000) Nestin expression in embryonic and adult human teeth under normal and pathological conditions. Am J Pathol 157:287-295

Arrigo AP, Préville X (1999) Role of Hsp27 and related proteins. In: Latchman DS (ed) Handbook of experimental pharmacology, vol 136. Stress proteins, chap 5. Springer, Berlin-New York, pp 101-132

Bronckers ALJJ, Lyaruu DM, Goei W, Litz M, Luo G, Karsenty G, Woltgens JH, D'Souza RN (1996) Nuclear DNA fragmentation during postnatal tooth development of mouse and hamster and during dentin repair in the rat. Eur J Oral Sci 104:102-111

Byers MR, Sugaya A (1995) Odontoblast processes in dentin revealed by fluorescent Di-I. J Histochem Cytochem 43:156-168

Frank RM, Steuer P (1988) Transmission electron microscopy of the human odontoblast process in peripheral root dentine. Arch Oral Biol 33:91-98

Fried K (1992) Changes in pulpal nerves with aging. Proc Finn Dent Soc 88:517-528

Goldberg M, Smith AJ (2004) Cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. Crit Rev Oral Biol Med 15:13-27

Gronthos S, Brahim J, Li W, Fisher L W, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81:531-535

Gunji T, Kobayashi S (1983) Distribution and organization of odontoblast processes in human dentin. Arch Histol Jpn 46:213-219

Holland GR (1990) Role of odontoblast process. In: Inoki R, Kudo T, Olgart L (eds) Dynamic aspects of dental pulp. Chapman and Hall, London, pp 73-95

Huot J, Houle F, Spitz DR, Landry J (1996) HSP27 phosphorylation-mediated resistance against actin fragmentation and cell death induced by oxidative stress. Cancer Res 56:273-279.

Izumi T, Kobayashi I, Okamura K, Matsuo K, Kiyoshima T, Ishibashi Y, Inoue H, Sakai H (1996) An immunohistochemical study of HLA-DR and alpha 1-antichymotrypsin-positive cells in the pulp of human non-carious and carious teeth. Arch Oral Biol 41:627-630

Izumi T, Inoue H, Matsuura H, Mukae F, Ishikawa H, Hirano H, Tamura N (2002) Age-related changes in the immunoreactivity of the monocyte/macrophage system in rat molar pulp after cavity preparation. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 94:103-110

Jakob U, Gaestel M, Engel K, Buchner J (1993) Small heat shock proteins are molecular chaperones. J Biol Chem 268:1517-1520

Jontell M, Gunraj MN, Bergenholtz G (1987) Immunocompetent cells in the normal dental pulp. J Dent Res 66:1149-1153

Jontell M, Bergenholtz G, Scheynius A, Ambrose W (1988) Dendritic cells and macrophages expressing class II antigens in the normal rat incisor pulp. J Dent Res 67:1263-1266

Jontell M, Jiang WH, Bergenholtz G (1991) Ontogeny of class II antigen expressing cells in rat incisor pulp. Scand J Dent Res 99:384-389

Jontell M, Bergenholtz G (1992) Accessory cells in the immune defense of the dental pulp. Proc Finn Dent Soc 88:344-355

Jontell M, Okiji T, Dahlgren U, Bergenholtz G (1998) Immune defense mechanisms of the dental pulp. Crit Rev Oral Biol Med 9:179-200

Kamal AM, Okiji T, Kawashima N, Suda H (1997) Defense responses of dentin/pulp complex to experimentally induced caries in rat molars: an immunohistochemical study on kinetics of pulpal Ia antigen-expressing cells and macrophages. J Endod 23:115-120

Kitamura C, Kimura K, Nakayama T, Toyoshima K, Terashita M (2001) Primary and secondary induction of apoptosis in odontoblasts after cavity preparation of rat molars. J Dent Res 80:1530-1534

Lavoie JN, Hickey E, Weber LA, Landry J (1993) Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J Biol Chem 268:24210-24214

Lovschall H, Fejerskov O, Josephsen K (2002) Age-related and site-specific changes in the pulpodentinal morphology of rat molars. Arch Oral Biol 47:361-367

Mehlen P, Schulze-Osthoff K, Arrigo A-P (1996) Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. J Biol Chem 271:16510-16514

Mehlen P, Mehlen A, Godet J, Arrigo A-P (1997) Hsp27 as a switch between differentiation and apoptosis in murine embryonic stem cells. J Biol Chem 272:31657-31665

Miron T, Vancompernolle K, Vandekerckhove J, Wilchek M, Geiger B (1991) A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J Cell Biol 114:255-261

Morse DR (1991) Age-related changes of the dental pulp complex and their relationship to systemic aging. Oral Surg Oral Med Oral Pathol 72:721-745

Murray PE, Stanley HR, Matthews JB, Sloan AJ, Smith AJ (2002) Age-related odontometric changes of human teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 93:474-482

Nakakura-Ohshima K, Watanabe J, Kenmotsu S, Ohshima H (2003) Possible role of immunocompetent cells and the expression of heat shock protein-25 in the process of pulpal regeneration after tooth injury in rat molars. J Electron Microsc 52:581-591

Nakasone N, Yoshie H, Ohshima H (2006) 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. Arch Oral Biol in press

Nanci A (2003) Dentin-pulp complex. In: Nanci A (ed) Ten Cate's Oral Histology: development, structure, and formation, 6th ed. Mosby, St Louis, pp 192-239

Ohshima H (1990) Ultrastructural changes in odontoblasts and pulp capillaries following cavity preparation in rat molars. Arch Histol Cytol 53:423-438

Ohshima H, Kawahara I, Maeda T, Takano Y (1994) 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

Ohshima H, Sato O, Kawahara I, Maeda T, Takano Y (1995) Responses of immunocompetent cells to cavity preparation in rat molars: an immunohistochemical study using OX6-monoclonal antibody. Connect Tissue Res 32:303-311

Ohshima H, Maeda T, Takano Y (1999) The distribution and ultrastructure of class II MHC-positive cells in human dental pulp. Cell Tissue Res 295:151-158

Ohshima H, Ajima H, Kawano Y, Nozawa-Inoue K, Wakisaka S, Maeda T (2000) Transient expression of heat shock protein (Hsp) 25 in the dental pulp and enamel organ during odontogenesis in the rat incisor. Arch Histol Cytol 63:381-395

Ohshima H, Nakakura-Ohshima K, Yamamoto H, Maeda T (2001a) Alteration in the expression of heat shock protein (Hsp) 25-immunoreactivity in the dental pulp of rat molars following tooth replantation. Arch Histol Cytol 64:425-437

Ohshima H, Nakakura-Ohshima K, Yamamoto H, Maeda T (2001b) Responses of odontoblasts to cavity preparation in rat molars as demonstrated by immunocytochemistry. Arch Histol Cytol 64:493-501

Ohshima H, Nakakura-Ohshima K, Maeda T (2002) Expression of heat shock protein (Hsp) 25-immunoreactivity in the dental pulp and enamel organ during odontogenesis in the rat molar. Connect Tissue Res 43: 220-223

Ohshima H, Nakakura-Ohshima K, Takeuchi K, Hoshino M, Takano Y, Maeda T (2003) Pulpal regeneration after cavity preparation, with special reference to close spatio-relationships between odontoblasts and immunocompetent cells. Microsc Res Tech 60:483-490

Okiji T, Kawashima N, Kosaka T, Matsumoto A, Kobayashi C, Suda H (1992) An immunohistochemical study of the distribution of immunocompetent cells, especially macrophages and Ia antigen-expressing cells of heterogeneous populations, in normal rat molar pulp. J Dent Res 71:1196-1202

Okiji T, Kosaka T, Kamal AM, Kawashima N, Suda H (1996) Age-related changes in the immunoreactivity of the monocyte/macrophage system in rat molar pulp. Arch Oral Biol 41:453-460

Rungvechvuttivittaya S, Okiji T, Suda H (1998) Responses of macrophage-associated antigen-expressing cells in the dental pulp of rat molars to experimental tooth replantation. Arch Oral Biol 43:701-710

Ruch JV, Lesot H, Begue-Kirn C (1995) Odontoblast differentiation. Int J Dev Biol 39:51-68

Sakurai K, Okiji T, Suda H (1999) Co-increase of nerve fibers and HLA-DR- and/or factor-XIIIa-expressing dendritic cells in dentinal caries-affected regions of the human dental pulp: an immunohistochemical study. J Dent Res 78:1596-1608

Shimizu A, Nakakura-Ohshima K, Noda T, Maeda T, Ohshima H (2000) Responses of immunocompetent cells in the dental pulp to replantation during the regeneration process in rat molars. Cell Tissue Res 302:221-233

Sigal MJ, Aubin JE, Ten Cate AR (1985) An immunocytochemical study of the human odontoblast process using antibodies against tubulin, actin, and vimentin. J Dent Res 64:1348-1355

Smith AJ (2002). Dentin formation and repair. In: Hargreaves KM, Goodis HE (eds) Seltzer and Bender's dental pulp. Quintessence Publishing, Chicago, pp 41-62

Suzuki T, Nomura S, Maeda T, Ohshima H (2004) An immunocytochemical study of pulpal responses to cavity preparation by laser ablation in rat molars by using antibodies to heat shock protein (Hsp) 25 and class II MHC antigen. Cell Tissue Res. 315:311-319

Tate Y, Yoshiba K, Yoshiba N, Iwaku M, Okiji T, Ohshima H (2006) Odontoblast responses to GaAlAs laser irradiation in rat molars: an experimental study using heat-shock protein-25-immunohistochemistry. Eur J Oral Sci 114:50-57

Ten Cate AR (1998) Dentin-pulp complex. In: Ten Cate AR (ed) Oral Histology: development, structure, and formation, 5th ed. Mosby, St Louis, pp 150-196

Terling C, Rass A, Mitsiadis TA, Fried K, Lendahl U, Wroblewski J (1995) Expression of the intermediate filament nestin during rodent tooth development. Int J Dev Biol 39:947-956

Thomas HF, Carella P (1984) Correlation of scanning and transmission electron microscopy of human dentinal tubules. Arch Oral Biol 29:641-646

Tsuchiya M, Sasano Y, Kagayama M, Watanabe M (2002) The extent of odontoblast processes in the dentin is distinct between cusp and cervical regions during development and aging. Arch Histol Cytol 65:179-188

Tsukamoto-Tanaka H, Ikegame M, Takagi R, Harada H, Ohshima H (2006) Histochemical and immunocytochemical study on hard tissue formation in dental pulp during the healing process after tooth replantation in rat molars. Cell Tissue Res in press

Yoshiba N, Yoshiba K, Nakamura H, Iwaku M, Ozawa H (1996) Immunohistochemical localization of HLA-DR-positive cells in unerupted and erupted normal and carious human teeth. J Dent Res 75:1585-1589

Yoshiba K, Yoshiba N, Ejiri S, Iwaku M, Ozawa H (2002) Odontoblast processes in human dentin revealed by fluorescence labeling and transmission electron microscopy. Histochem Cell Biol 118:205-212

Figure legends

Fig. 1. Goodpasture Gram staining in the injured tooth after 12 h from the aged rat. a. The marked aggregation of Gram-positive bacteria (*) is observed in the prepared cavity (*C*). b. Higher magnification of the bacteria mass in the prepared cavity. Gram-positive cocci were recognized in the mass (*arrows*). c. Higher magnification of the boxed area labeled by *c* in a. Numerous irregular shaped cells (*arrows*) aggregate along the pulp-dentin border. There are no Gram-positive bacteria in the dental pulp. *D* dentin, *DP* dental pulp, *bars* 100 μm (a), 25 μm (c), 10 μm (b).

Fig. 2. HSP-25- (a, c, e) and OX6-immunoreactivities (b, d, f) in the control (a, b) and injured teeth after 12 h (c, d) and 3 d (e, f) from the young adult rats. a. Intense immunoreaction for HSP-25 is found in the odontoblasts, but not in the odontoblast processes except at their bottoms within the predentin. b. OX6-immunopositive cells are located predominantly beneath the odontoblast layer and extend their processes between the odontoblasts (*arrow*). c. HSP-25-immunoreactive cells are recognized beneath the degenerated odontoblast layer. d. OX6-immunopositive cells accumulate along the pulp-dentin border and extend their processes deep into the dentinal tubules (*arrows*). e. HSP-25-immunoreactive odontoblast-like cells are arranged in the proper odontoblast layer. f. OX6-immunopostive cells are located beneath the newly differentiated odontoblast-like cells. *D* dentin, *DP* dental pulp, *OB* odontoblasts or odontoblast-like cells, * odontoblast layer that has suffered damage, *bars* 50 μm.

Fig. 3. HSP-25- (a, c, e), OX6- (b, d, f) and nestin-immunoreactivity (IR) (g, h) in the control teeth from the aged rats. a. The progressive attrition and considerable cementum deposition (*arrows*) are recognized as age-related changes, resulting in the shallow occlusal grooves (*arrowheads*). b. OX6-immunopositive cells are densely distributed in the coronal dental pulp. c. Higher magnification of the boxed area labeled by *c* in a. Corresponding to the enamel-free area, tertiary dentin formation is observed (*). Intense IR for HSP-25 is recognizable in the odontoblasts except for the weak positive cells in the pulp floor (*arrows*), and some nerve fibers and blood vessels show weak HSP-25-immunoreaction. d. Higher magnification of the boxed area labeled by *d* in b. OX6-immunopositive cells are predominantly located at the periphery of the pulp tissue. Most of the subodontoblastic immunopositive cells appear to have a dendritic shape, and some are located in the predentin (*arrows*). e. Higher magnification of the boxed area labeled by *e* in c. Odontoblasts in the coronal pulp are intensely immunoreactive for HSP-25 in their cytoplasm. Their cell processes in the predentin are seen to be immunoreactive. f. Higher magnification of the boxed area labeled by *f* in b. OX6-immunopositive cells in the periphery are considerably decreased in number in the root region. The red dotted lines show the boundary between the crown and root. g. Intense nestin-IR is exclusively observed in the coronal odontoblasts and their processes (*arrowheads*). h. The odontoblasts and their processes in the pulp floor also show intense nestin-IR, although these cells lack cell continuity in addition to losing the subodontoblastic cell-rich zone. *AB* alveolar bone, *D* dentin, *DP* dental pulp, *OB* odontoblasts, *bars* 500 μm (a, b), 200 μm (c), 50 μm (d, e, f), 25 μm (g, h).

Fig. 4. HSP-25- (a, c, e, g, i) and OX6-immunoreactivities (b, d, f, h, j) in the injured teeth after 0 (a-d), 12 (e, f) and 24 h (g, h), and 3d (i, j) from the aged rats. a. The odontoblasts under the prepared cavity suffer severe damage, whereas the intact cells retain intense HSP-25-IR. b. OX6-immunopositive cells shift inwards together with the odontoblasts that have suffered damage. c. Higher magnification of the boxed area labeled by *c* in b. The cells, round in shape without cell processes, retain the Hsp 25-IR. d. Higher magnification of the boxed area labeled by *d* in a. OX6-immunopositive cells still display a dendritic appearance at this region. e. Most immunoreactive cells appear round in shape. f. OX6-immunopositive cells accumulate along the pulp-dentin border and extend their processes deep into the dentinal tubules (*arrows*). g. No apparent immunoreaction for HSP-25 persists at the pulp-dentin border, whereas HSP-25-IR remains in the subodontoblastic layer apart from the predentin. h. OX6-immunopositive cells remain along the pulp-dentin border, but are reduced in number from the previous stage. i. HSP-25-immunoractive odontoblast-like cells line up in the proper odontoblast layer. j. OX6-immunopostive cells are located beneath the newly differentiated odontoblast-like cells. The red dotted lines show the boundary between the dentin that has suffered damage and the intact dentin. *C* prepared cavity, *D* dentin, *DP* dental pulp, *OB* odontoblasts or odontoblast-like cells, * odontoblast layer that has suffered damage, *bars* 200 μm (a, b), 50 μm (c-j).

Fig. 5. Cryo- (a, b), paraffin- (c, d) and semithin-sections (e, f) and electron micrographs (g, h) of HSP-25- (a, e-h), OX6- (b) and nestin-IR (c, d) in the mild (a, b, c, e, g) and severe (d, f, h) cases of injured teeth after 6 h (e, g) and 12 h (a-d, f, h) from the aged rats. a. The odontoblast layer shows no changes in the IR for HSP-25. b. Numerous OX6-immunopositive cells aggregate at the pulp-dentin border and extend their cellular processes into the dentinal tubules. c. The odontoblasts and their processes retain nestin-IR even in the area that suffered damage. d. No intense nestin-IR is recognized in the odontoblasts and their processes in the area that suffered damage. e. HSP-25-immunopositive odontoblasts retain their cell layer. f. Numerous HSP-25-immunonegative cells appear along the pulp-dentin border. g. The odontoblasts retain immunoreaction for HSP-25 in their cytoplasm, but a presumably dendritic cell with a tubulovesucular structure and a polymorphonuclear leukocyte are observed in the pulp-dentin border. h. A presumably dendritic cell with tubulovesicular structures and a polymorphonuclear leukocyte are located at the pulp-dentin border, and odontoblasts that have suffered damage showing a round appearance maintain their IR for HSP-25. *D* dentin, *OB* odontoblasts or odontoblast-like cells, *PD* predentin, *PML* polymorphonuclear leukocyte * presumably dendritic cell, *bars* 50 μm (a-d), 25 μm (e, f), 5 μm (g, h).

Fig. 6. Comparison between the OX6-immunoreactive fractions at the periphery of young adult pulp and those of aged pulp in the control and injured teeth after 12 h and 3 d. The fractions of immunoreactive area in aged molars were significantly

larger than those in young adult molars in the control and 3 d after cavity preparation.

Fig. 7. A schematic diagram indicating the possible hypothesis concerning the pulpal reactions to cavity preparation in the aged tooth. Cavity preparation (a) caused the two types of pulpal reactions showing the different extent of damage in the early stages: severe (b, c) and mild damage (d, e). A variety of reactions in aged teeth under the current experimental model may be attributed to differences in the status of dentinal tubules and/or odontoblast processes in individuals. In the case of severe damage (b, c), most odontoblasts that suffered damage disappear from the pulp-dentin border and numerous dendritic cells accumulate at the pulp-dentin border and extend their processes into the dentinal tubules. In the case of mild damage (d, e), some odontoblasts are degenerated and the dendritic cells appear at the pulp-dentin border. Individual odontoblasts may suffer a variety of damage depending on the length of their cellular processes. *DC* dendritic cells, *OB* odontoblasts.