Microsc. Res. Tech. Submitted 2002

Pulpal Regeneration after Cavity Preparation, with Special Reference to Close Spatio-relationships between Odontoblasts and Immunocompetent Cells

Hayato Ohshima[1], Kuniko Nakakura-Ohshima[2], Kiichi Takeuchi[3], Masaaki Hoshino[3], Yoshiro Takano[4] and Takeyasu Maeda[3]

Division of Anatomy and Cell Biology of the Hard Tissue[1], Department of Tissue Regeneration and Reconstruction, and Division of Oral Anatomy[3], Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, Polyclinic Intensive Oral Care Unit[2], Niigata University Dental Hospital, and Department of Hard Tissue Engineering[4], Graduate School of Tokyo Medical and Dental University, Japan

Running title: Pulpal Responses to Cavity Preparation

Key Words. class II MHC molecule, dental pulp, heat shock protein 25, tissue reaction

Address for correspondence:
Hayato Ohshima, DDS, PhD
Division of Anatomy and Cell Biology of the Hard Tissue
Department of Tissue Regeneration and Reconstruction
Niigata University Graduate School of Medical and Dental Sciences
2-5274 Gakkocho-dori, Niigata
951-8514, Japan
TEL +81-25-227-2812
FAX +81-25-227-0804
E-mail: histoman@dent.niigata-u.ac.jp

Abstract

The regeneration process of the odontoblast cell layer incident to tooth injury, especially its relationship with immunocompetent cells in pulp healing, has not been fully understood. The purpose of the present study was to clarify this relationship between odontoblasts and immunocompetent cells in the process of pulp regeneration following cavity preparation in rat molars by immunocytochemistry for heat shock protein (Hsp) 25 as well as class II major histocompatibility complex (MHC) molecules. In untreated control teeth, intense Hsp 25-immunoreactivity was found in the cell bodies of odontoblasts and their processes within the predentin, whereas class II MHC-positive cells were predominantly located beneath the odontoblast cell layer. Cavity preparation caused the destruction of the odontoblast layer to form an edematous lesion and the shift of class II MHC-positive cells with the injured odontoblasts toward the pulp core at the affected site. Some damaged odontoblasts without apparent cytoplasmic processes, round in profile, retained the immunoreactivity for Hsp25, suggesting the survival of a part of the odontoblasts against artificial external stimuli. Twelve hours after cavity preparation, numerous class II MHC-positive cells appeared along the pulp-dentin border and extended their processes deep into the exposed dentinal tubules. By postoperative 72 hours, newly-differentiated odontoblasts with Hsp 25-immunoreactivity were arranged at the pulp-dentin border, but the class II MHC-positive cells moved from the pulp-dentin border to the subodontoblastic layer. These findings indicate

that the time course of changes in the expression of Hsp 25-immunoreactivity reflects the regeneration process of odontoblasts. The functional roles of Hsp 25-positive odontoblasts and immunocompetent cells such as class II MHC-positive cells in the process of pulp regeneration after cavity preparation are discussed in conjunction with our previous experimental data.

Introduction

The tooth is often exposed to dental caries, attrition, abrasion and restorative procedures such as cavity preparation to cause injury to dental pulp. The procedure of cavity preparation induces destructive changes in odontoblasts at the affected site as well as an acute inflammatory reaction (Eda and Saito, 1978; Lilja et al., 1982; Mjör, 1983; Seltzer and Bender, 1984; Hirvonen and Närhi, 1986; Sato, 1989; Ohshima, 1990). If the odontoblasts survive, they are capable of depositing further reactionary dentin, i.e. reparative dentin. If not, pulpal mesenchymal cells take the place of the degenerated odontoblasts to differentiate into new odontoblasts (Ten Cate, 1998). However, the regeneration process of the odontoblast cell layer has not been fully understood, one of reasons being the lack of any reliable marker substance for differentiation of odontoblasts until recently.

Heat shock protein (Hsp) 25, the family of low molecular weight Hsps, is expressed in normal various cells as well as under stressful conditions, although it was first discovered under the latter conditions (for reviews, see Ciocca, 1993; Arrigo and Préville, 1999). This protein has been reported to possess diverse functions such as molecular chaperones (Jacob et al., 1993), or modulating actin dynamics to protect cell motility and shape (Lavoie et al., 1993b; Mariesse et al., 1996), a specific inhibitor of apoptosis (Mehlen et al., 1997) and promoter of cell survival (Huot et al., 1991; Lavoie et al., 1993a; Mehlen et al., 1995, 1996; Wu and Welsh, 1996). In addition to stressful and normal conditions, the transient

expression of Hsp 25 has been shown during cellular development and differentiation (Arrigo and Préville, 1999). Our recent studies have demonstrated a stage-specific expression pattern of Hsp 25-immunoreactivity in the odontoblasts under normal (Ohshima et al., 2000a, 2002) and experimental conditions (Ohshima et al., 2001a, b), suggesting that this protein is a useful marker for the differentiation of odontoblasts during the pulpal healing process.

The dental pulp, a loose connective tissue surrounded by dental hard tissues, contains many immunocompetent cells that serve in an initial defense reaction and for antigen-presentation (for review, see Jontell et al., 1998). Although many kinds of immunocompetent cells have been confirmed in dental pulp (Ten Cate, 1998), the most abundant one is the cell with the class II major histocompatibility complex (MHC) antigen. The class II MHC antigen is called Ia antigen in the rat, and can be recognized by an OX6 antibody (McMaster and Williams, 1979). To date, these OX6-immunopositive cells have been reported to show characteristic reaction patterns under various experimental conditions such as tooth grinding (Ohshima et al., 1995, 1996; Kamal et al., 2000), tooth replantation (Rungvechvuttivittaya et al., 1998; Shimizu et al., 2000), and carious teeth (Yoshiba et al., 1996; Izumi et al., 1996; Kamal et al., 1997; Sakurai et al., 1999). Interestingly, under experimental conditions, the class II MHC-positive cells have been suggested to have diverse functions in odontoblast differentiation in the rat dental pulp in addition to ordinary antigen presentation (Ohshima et al., 1995). Under physiological

conditions, furthermore, class II MHC-positive cells in the human odontoblast layer and/or predentin might have some regulatory function in the homeostasis of odontoblasts (Ohshima et al., 1999). These findings therefore lead us to the possibility that the immunocompetent cells play an important role in the process of pulpal healing after cavity preparation.

In our previous studies (Ohshima et al., 1995, 2001b), we were able to demonstrate the differentiation process of odontoblasts during the pulpal healing process incident to cavity preparation. However, the relationship between odontoblasts and immunocompetent cells remains unclear. The purpose of the present study is to clarify the relationship between odontoblasts and immunocompetent cells following cavity preparation in the rat molars by immunocytochemistry for Hsp 25 and Ia antigen.

Materials and Methods

All experiments were performed following the Guidelines of the Niigata University Intramural Animal Use and Care Committee.

Twenty-five Wistar rats, 100 days old, were used in this study. Under anesthesia by an inhalation of 3% Halothane gas, a groove-shaped cavity (the width of the remaining dentin being around 150–200 μm) was prepared on the mesial surface of the upper right first molar by use of an air turbine with a tungsten carbide bur (diameter 0.5 mm) under water-cooling. The cavity was left without any further treatment such as air-drying, etching or filling. The upper left first molar of the same animal was used as a control.

Materials were collected from groups of five animals at intervals of 0, 6, 12, 24, and 72 hours 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 hours. Following decalcification in a 5% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution for 4 weeks at 4 °C, the tissue blocks were equilibrated in a 30% sucrose solution for cryoprotection. The specimens were cut sagittally at a thickness of about 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.

For the immuno-peroxidase procedure, frozen sections were processed for the avidin-biotin peroxidase complex (ABC) method (Hsu et al. 1981) using either a polyclonal antibody to Hsp 25 (diluted 1:5000; StressGen Biotechnologies Corp., Victoria, BC, Canada) or an OX6-monoclonal antibody (diluted 1:5000; Serotic Ltd., Oxford, UK). An anti-Hsp 25 antibody can recognize Hsp 25/27 from the mouse, hamster, guinea pig, rat, bovine and canine cell lysates, and an OX6 antibody recognizes class II MHC molecules from the rat. A solution of 0.01 M phosphate-buffered saline (PBS, pH 7.4) was used both to dilute the antibody and to rinse the sections. Endogenous peroxidase was inactivated by treatment with 0.3% normal horse serum (Vector Lab. Inc., Burlingame, CA) for an OX6 antibody or 2.5% normal goat serum (Vector) for an anti-Hsp 25 antibody. Following incubation with one of the primary antibodies for 3 days at 4°C, the sections were consecutively reacted either with biotinylated anti-mouse IgG (for an OX6 antibody; Vector) or biotinylated anti-rabbit IgG (for an anti-Hsp 25 antibody; Vector) for 24 hours at 4°C, followed by the ABC complex (Vector) for 2 hours at room temperature. The antigen-antibody reaction sites were made visible by 0.04% 3,3'-diaminobenzidine tetrachloride and 0.002% H_2O_2 in a 0.05 M Tris HCl buffer (pH 7.6). Immunostained sections were thaw-mounted onto silane-coated glass slides, and stained with 0.03% methylene blue.

For immunocytochemistry using an OX6-monoclonal antibody at the electron-microscopic level, the immunostaining procedure was the

same as described above, except for the inhibition of endogenous 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). Ultrathin sections (70 nm in thickness) were double-stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7000 transmission electron microscope.

Immunohistochemical controls were performed by: 1) replacing the primary antibodies with non-immune serum or PBS; 2) omitting the anti-rabbit IgG, the anti-mouse IgG or the ABC complex. These immunostained sections did not contain any specific immunoreaction. Further characterization of the primary antibodies has been reported elsewhere (Plumier et al., 1997; Ohshima et al., 2000a).

Results

Controls

Hsp 25-immunohistochemistry in the dental pulp of rat molars demonstrated an intense immunoreaction in the odontoblasts, but a weak one in other cellular elements including nerve fibers and blood vessels. The immunoreaction within odontoblasts comprised the cell bodies and cell processes limited within the predentin (Fig. 1a). No immunonegative odontoblast was found in the control teeth. Many OX6-immunopositive cells were widely distributed throughout the dental pulp of rat molars, predominantly in the periphery of the pulp tissue. Most of the OX6-immunopositive cells in the subodontoblastic region exhibited dendritic profiles, and some of them extended their processes into the odontoblast layer (Fig. 2a). The pulpo-dentinal border zone including the odontoblast cell layer contained few OX6-positive cells in the untreated control teeth (Fig. 2a). Electron microscopic observations confirmed electron-opaque immunoreactive products for Ia antigen in the cell membrane (Fig. 3a). These immunopositive cells were ultrastructurally characterized by a well-developed Golgi apparatus, mitochondria, tubulovesicular structures and multivesicular bodies (Fig. 3b). The odontoblasts did not have any OX-6 immunoreactions (Fig. 3a).

Immediately after cavity preparation

Cavity preparation rapidly induced an exudative lesion between the predentin and impaired odontoblast layer (Figs. 1b, 2b). The odontoblasts

under the prepared cavity suffered severe damage and a diminished Hsp 25-immunoreaction. The impaired odontoblasts without an Hsp 25-immunoreaction and the Hsp 25-positive cells without apparent cell processes were intermingled in the damaged odontoblast cell layer. Cell fragments showing Hsp 25-immunoreactivity also remained in the predentin beneath the damaged dentin (Fig. 1b). OX6-immunopositive cells showing a dendritic appearance at the affected site shifted inwards together with the separated and damaged odontoblasts (Fig. 2b).

6 hours after cavity preparation

The damaged odontoblasts with Hsp 25-immunoreaction came to exhibit rounded profiles in the impaired odontoblast layer due to the loss of their cytoplasmic processes, although some cells retained their Hsp 25-immunoreactive cell processes. Hsp 25-immunonegative cells were situated along the pulp-dentin border (Fig. 1c). In OX6-immunohistochemistry, some immunopositive cells were located in the vicinity of the predentin and extended their processes toward the exposed dentinal tubules (Fig. 2c). At the ultrastructural level, the dentinal tubules contained the cell debris of degenerated odontoblasts showing vesiculated features in the cytoplasm and the pyknotic nucleus. Both OX6-positive and negative cells including numerous phagosomes, presumably both class II MHC-positive and negative macrophages phagocytizing degenerated odontoblasts, were located along the

pulp-dentin border (Fig. 4). Polymorphonuclear leukocytes were also frequently recognized at the pulp-dentin border.

12 hours after cavity preparation

The pulp-dentin border completely lost the immunoreactivity for Hsp 25, in spite of the occurrence of some cells there. Hsp 25-immunoreactive cells were discernible beneath the exudative lesions (Fig. 1d). In contrast, OX6-immunopositive cells accumulated at the affected area along the pulp-dentin border; they frequently extended their processes deep into the dentinal tubules (Fig. 2d). Transmission electron microscopic observations revealed that such immunopositive cells extending cytoplasmic processes in the dentinal tubules possessed characteristic tubulovesicular structures, multivesicular bodies, and vacuoles (Fig. 5). On the other hand, the distribution pattern and shape of OX6-positive cells at the other intact site were the same as those at the previous stage.

24 hours after cavity preparation

The exudative lesions had almost disappeared by 24 hours after cavity preparation. No apparent immunoreaction for Hsp 25 persisted in the pulp-dentin border, whereas a weak Hsp 25-immunoreactivity was found in some mesenchymal cells at the area apart from the predentin (Fig. 1e). OX6-immunopositive cells extending their processes into the dentinal tubules remained along the pulp-dentin border, but were decreased in number from the previous stage (Fig. 2e).

72 hours after cavity preparation

The Hsp 25-immunoreactive plump cells, presumably newly-differentiated odontoblasts, lined up in the proper odontoblast layer (Fig. 1f). OX6-immunopositive cells with dendritic profiles were exclusively located beneath the newly-differentiated odontoblasts (Fig. 2f). These differentiated odontoblasts contained a well-developed Golgi apparatus and rough-surfaced endoplasmic reticulum. The OX6-immunopositive cells showed the same cytoplasmic features as those of the cells beneath the intact odontoblasts layer in the control teeth (Fig. 6).

Figure 7 summarizes the spatiotemporal relationship between class II MHC-positive cells and Hsp 25-positive odontoblasts in the pulp-dentin border following cavity preparation.

Discussion

In the present immunocytochemical study, cavity preparation caused drastic time-related alterations in Hsp 25 expression in the odontoblasts in degeneration/regeneration process in rat molars. Furthermore, the class II MHC-positive cells dramatically changed their locations and populations in correlation with the degeneration/regeneration of the odontoblast layer.

An abundant distribution of class II MHC-positive cells has been reported repeatedly in the dental pulp, including rats under normal conditions (Okiji et al., 1992, Ohshima et al., 1994, 1995). It includes dendritic cells and macrophages. The cells with class II MHC-antigen participate in the initial recognition and the processing of antigenic substances to serve as antigen-presenting cells. Following exposure to antigens, they migrate to the lymphoid tissue via afferent lymph to activate T-cells and induce their cell proliferation (for review, Steinman, 1991; Thery and Amigorena, 2001). In general, dendritic cells are believed to have a higher potential for antigen-presentation than macrophages which serve as scavengers as well as for antigen-presentation. Histochemical and immunohistochemical studies (Ohshima et al., 1994, 1997) have demonstrated the localization of only weak acid phosphatase (ACPase) activity in the odontoblast layer and a lack of typical phagosomes in the class II MHC-positive cells situated deep in the odontoblast layer. Although the dental pulp has been known to contain class II MHC-positive macrophages which eliminate degenerated odontoblasts (Ohshima et al., 1994), we can regard the class II MHC-positive cells in the odontoblast

layer in control teeth as dendritic cells without any phgocytotic activity, as supported by the present electron microscopic observation; class II MHC-positive cells beneath the odontoblast layer had no phagosomes but multivesicular bodies and fine tubulovesicular structures.

It is noteworthy that a temporal aggregation of class II MHC-positive cells appeared at the pulp-dentin border 12 – 24 hours after cavity preparation in the rat molar. These class II MHC-positive cells along the pulp-dentin border were characterized by tubulovesicular structures and multivesicular bodies, as categorized in the pulpal dendritic cells described above (Ohshima et al., 1994, 1995, 1999). This phenomenon may be explained by the idea that the pulpal dendritic cells respond actively to bacterial or noxious substances derived through the exposed dentinal tubules, as we have suggested (Ohshima et al., 1995). However, similar findings have been also reported even in a tooth replantation model using rat molars whose dentinal tubules are not exposed to the oral environment (Shimizu et al., 2000). Furthermore, class II MHC-positive cells almost disappeared from the odontoblast layer after the deposition of reparative dentin on the preoperative dentin in cavity preparation (present study; Ohshima et al., 1995) and tooth replantation models (Shimizu et al., 2000). These experimental data imply the putative role of class II MHC-positive cells in odontoblast differentiation during pulpal regeneration, based on a temporal appearance of the class II MHC-positive cells along the pulp-dentin border during pulp regeneration after tooth injury. This may be supported by our recent experimental data

showing that the same temporal accumulation of class II MHC-positive cells occurs during the regeneration process of the infected pulp following the application of antimicrobials, but never in the case of abscess formation (Ohshima et al., 2000b).

There is no doubt that cavity preparation injured the odontoblasts to some degree. Many researchers agree with the notion that pulpal mesenchymal cells near the subodontoblastic capillaries migrate to the damaged odontoblast layer to differentiate into new odontoblasts there (Sveen and Hawes, 1968; Searls, 1975; Mjör, 1983; Ohshima, 1990). However, there has been controversy over the fate of the damaged odontoblasts. Our previous study demonstrated that fragments of the destroyed odontoblasts such as rounded nuclei and cell organelles were scattered throughout the exudative lesions immediately after cavity preparation, and that these damaged odontoblasts degenerated immediately after surgery (Ohshima, 1990), as reported by Bronkers et al. (1996) and Kitamura et al. (2001). This finding is supported by the present electron microscopic observations of the degenerated cell debris in the dentinal tubules postoperative 6 hours. They are likely phagocytized by both class II MHC-positive and negative macrophages as well as polymorphonuclear leukocytes. In contrast, Chiego (1992) pointed out the possibility that the affected odontoblasts could survive and deposit reparative dentin after cavity preparation. In a recent study of ours (Ohshima et al., 2001b), some impaired odontoblasts did survive in the injured odontoblast layer, though most of them showed degenerated features. The alterations in the

expression of Hsp 25-immunoreactivity in the odontoblasts as shown in this study seems to well reflect the status of their degeneration/regeneration process; the injured odontoblasts at the pulp-dentin border lost the Hsp 25-immunoreactivity at postoperative 6 hours, but they retained their immunoreaction beneath the edematous lesions 12 hours after the cavity preparation. Kitamura et al. (2001) have revealed two waves of apoptosis in the injured odontoblasts after cavity preparation: the primary induction of apoptosis appears in the odontoblasts at postoperative 1 hour, and subsequently in the subodontoblastic region at postoperative 1 day. The time course of the occurrence of their apoptosis appear to correlate with the alterations in Hsp 25-immunoreaction in the odontoblasts. Taken together, these facts would indicate that even the surviving odontoblasts escaping from the primary degeneration are fated to be ultimately degenerated.

The functional significance of consistent Hsp 25 expression in odontoblasts remains unknown. Our previous observations showed constant and intense Hsp 25-immunoreaction in the differentiated odontoblasts in the incisor pulp and the coronal pulp of the molar. Moreover, the odontoblasts in the root and floor pulp of the molar were initially weak or negative, but increased in Hsp 25-immunointensity during the later stages of development (Ohshima et al., 2000a, 2002). These findings indicate the possibility that Hsp 25-immunoreaction is a useful marker for distinguishing between undifferentiated and differentiated odontoblasts, as Hsp 25 has been reported to accumulate in the early phase of differentiation concomitantly with a decreased rate of cellular

proliferation (Mehlen et al., 1997). This idea is strongly supported by the present and previous experimental findings that newly-differentiated odontoblasts acquire Hsp 25-immunoreactivity following cavity preparation (present study; Ohshima et al., 2001b) and tooth replantation (Ohshima et al., 2001a). Recently, Wells and Malkovsky (2000) have hypothesized that Hsp functions as a general mediator of inflammation; a stress response such as hypoxia, radiation or chemotherapy in tumor cells results in the up-regulation of intracellular Hsp, and the subsequent extracellular filtration of their Hsp contents – induced by the tumor cell death – activates neighboring polymorphonuclear leukocytes and recruits antigen-presenting cells. Therefore, it may be more suitable to say that the high levels of intracellular Hsp 25 in the odontoblasts serves in the rapid recruitment of immunocompetent cells such as class II MHC-positive cells after their complete destruction. Indeed, a close spatio-relationship between the immunocompetent cells and odontoblasts to cavity preparation has been demonstrated in cavity preparation (present study; Ohshima et al., 1995) and tooth replantation models (Shimizu et al., 2000).

Acknowledgments

This work was supported by a Grant for Promotion of Niigata University Research Projects and by Grants-in-Aid for Scientific Research (to promote 2001-Multidisciplinary Research Projects in 2001-2005; nos. 12671765 and 14571727 to H.O. and 13672141 to K.N.-O.) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

Arrigo, A.P., and Préville, X. (1999) Role of Hsp 27 and related proteins. In: Handbook of experimental pharmacology, Vol. 136. Stress proteins, Chap. 5. D. S. Latchman, ed. Springer, Berlin – New York, pp. 101-132.

Bronckers, A.L.J.J., Lyaruu, D.M., Goei, W., Litz, M., Luo, G., Karsenty, G., Woltgens, J.H., and D'Souza, R.N. (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.

Chiego, D.J.Jr. (1992) An ultrastructural and autoradiographic analysis of primary and replacement odontoblasts following cavity preparation and wound healing in the rat molar. Proc. Finn. Dent. Soc., 88:243-256.

Ciocca, D.R., Oesterreich, S., Chamness, G.C., McGuire, W.L., and Fuqua, S.A.W. (1993) Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. J. Natl. Cancer Inst., 85:1558-1570.

Eda, S., and Saito, T. (1978) Electron microscopy of cells displaced into the dentinal tubules due to dry cavity preparation. J. Oral Pathol., 7:326-335.

Hirvonen, T.J., and Närhi, M.V. (1986) The effect of dentinal stimulation on pulp nerve function and pulp morphology in the dog. J. Dent. Res., 65:1290-1293.

Hsu, S.M., Raine, L., and Fanger, H. (1981) 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.

Huot, J., Roy, G., Lambert, H., Chrétien, P., and Landry, J. (1991). Increased survival after treatments with anticancer agents of Chinese hamster cells expressing the human Mr 27,000 heat shock protein. Cancer Res., 51:5245-5252.

Izumi, T., Kobayashi, I., Okamura, K., Matsuo, K., Kiyoshima, T., Ishibashi, Y., Inoue, H., and 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.

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

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

Kamal, A.M.M., Okiji, T., Kawashima, N., and 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.

Kamal, A.M.M., Okiji, T., and Suda, H. (2000) Response of Class II molecule-expressing cells and macrophages to cavity preparation and restoration with 4-META/MMA-TBB resin. Int. Endod. J., 33:367-373.

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, J.N., Gingras-Breton, G., Tanguay, R.M., Landry, J. (1993a) Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. HSP27 stabilization of the microfilament organization. J. Biol. Chem., 268:3420-3429.

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

Lilja, J., Nordenvall, K.J., and Brännström, M. (1982) Dentin sensitivity, odontoblasts and nerves under desiccated or infected experimental cavities. A clinical, light microscopic and ultrastructural investigation. Swed. Dent. J., 6:93-103.

Mairesse, N., Horman, S., Mosselmans, R., and Galand, P. (1996) Antisense inhibition of the 27 kDa heat shock protein production affects growth rate and cytoskeletal organization in MCF-7 cells. Cell Biol. Int. 20:205-212.

McMaster, W.R., and Williams, A.F. (1979) Identification of Ia glycoproteins in rat thymus and purification from rat spleen. Eur. J. Immunol., 9:426-433.

Mehlen, P., Preville, X., Chareyron, P., Briolay, J., Klemenz, R., and Arrigo, A.P. (1995) Constitutive expression of human hsp27, Drosophila hsp27, 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.

Mehlen, P., Kretz-Remy, C., Preville, X., and Arrigo, A.P. (1996) Human hsp27, Drosophila hsp27 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., 15:2695-2506.

Mehlen, P., Mehlen, A.A., Godet, J., and Arrigo, A.P.: Hsp 27 as a switch between differentiation and apoptosis in murine embryonic stem cells. J. Biol. Chem., 272:31657-31665.

Mjör, I.A. (1983) Dentin and pulp. In: Reaction patterns in human teeth, I. A. Mjör, ed. CRC Press Inc., Boca Raton, Florida, pp. 63-156.

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., and 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., and 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., Takano, Y., Sato, O., Kawahara, I., and Maeda, T. (1996) Responses of class II MHC antigen-expressing cells to cavity preparation. In: Dentin Pulp Complex: Proceeding of the International Conference on Dentin Pulp Complex 1995, M. Shimono, T. Maeda, H. Suda, and K. Takahashi, eds. Quintessence, Tokyo, pp. 316-318.

Ohshima, H., Maeda, T., and Takano, Y. (1997) Subpopulation of class II MHC antigen-expressing cells in the rat incisor pulp as shown by acid phosphatase histochemistry. Dentistry Jpn., 33:8-14.

Ohshima, H., Maeda, T., and 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., and Maeda, T. (2000a) 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., Sato, T., Takahashi, N., and Maeda, T. (2000b) Response of the infected pulp to antimicrobials in rat molars (abstract). J. Dent. Res., 79:1237.

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

Okiji, T., Kawashima, N., Kosaka, T., Matsumoto, A., Kobayashi, C., and 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.

Plumier, J.C., Hopkins, D.A., Robertson, H.A., and Currie, R.W. (1997) 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.

Rungvechvuttivittaya, S., Okiji, T., and 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.

Sakurai, K., Okiji, T., and 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.

Sato, O. (1989) Responses of pulpal nerves to cavity preparation in rat molars: an immunohistochemical study using neurofilament protein (NFP) antiserum. Arch. Histol. Cytol., 52:433-446.

Searls, J.C. (1975) Radioautographic evaluation of changes induced in the rat incisor by high-speed cavity preparation. J. Dent. Res., 54:174-182.

Seltzer, S., and Bender, I.B. (1984) Pulpitis from operative procedures. In: The dental pulp. Biological considerations in dental procedures. 3rd ed. S. Seltzer and I. B. Bender, eds. J. B. Lippincott Co., Philadelphia – London – New York – St. Louis – São Paulo – Sydney, pp. 252-273.

Shimizu, A., Nakakura-Ohshima, K., Noda, T., Maeda, T., and 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.

Steinman, R.M. (1991) The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol., 9:271-296.

Sveen, O.B., and Hawes, R.R. (1968) Differentiation of new odontoblasts and dentine bridge formation in rat molar teeth after tooth grinding. Arch. Oral Biol., 13:1399-1409.

Ten Cate, A.R. (1998). Oral Histology: development, structure, and formation. 5th ed. Mosby, St. Louis – Baltimore – Boston – Chicago – London – Madrid – Philadelphia – Sydney – Toronto, pp. 1-497.

Thery, C., and Amigorena, S. (2001) The cell biology of antigen presentation in dendritic cells. Curr. Opin. Immunol., 13:45-51.

Vermelin, L., Lecolle, S., Septier, D., Lasfargues, J.J., and Goldberg, M. (1996) Apoptosis in human and rat dental pulp. Eur. J. Oral Sci., 104:547-553.

Wells, A.D., and Malkovsky, M. (2000) Heat shock proteins, tumor immunogenicity and antigen presentation: an integrated view. Immunol. Today., 21:129-132.

Wu, W., and Welsh, M.J. (1996) Expression of the 25-kDa heat-shock protein (HSP25) 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 HSP25 cDNA. Toxicol. Appl. Pharmacol., 141:330-339.

Yoshiba, N., Yoshiba, K., Nakamura, H., Iwaku, M., and 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.

Figure legends

Fig. 1. Hsp25-immunoreactivities in control (**a**) and injured rat molars (**b–f**). **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.** Immediately after operation. Cavity preparation induces exudative lesions between the predentin and damaged odontoblast layer. The round cells without any apparent cell processes retain Hsp 25-immunoreactivity. Note Hsp 25-immunoreactivity in the cell fragments at the pulp-dentin border (*arrows*). **c.** 6 hours after operation. The impaired odontoblasts both with and without their slender processes retain immunoreactivity for Hsp 25. **d.** 12 hours after operation. The Hsp 25-immunoreactive cells are recognized beneath the degenerated odontoblast layer. The exudative lesion remains in the dental pulp beneath the injured dentin. Hsp 25-immunonegative cells align along the pulp-dentin border (*arrows*). **e.** 24 hours after operation. The exudative lesion has almost diminished, but the pulp-dentin border lacks immunoreaction for Hsp 25. Hsp 25-immunonegative cells remain at the pulp-dentin border (*arrows*), but some mesenchymal cells represent weak immunoreaction at the area apart from the predentin (*arrowheads*). **f.** 72 hours after operation. The Hsp 25-immunoreactive plump cells, presumably newly-differentiated odontoblasts, are arranged in the proper odontoblast layer. *D* dentin,

DP dental pulp, *OB* odontoblasts, * exudative lesion enhanced artificially during tissue preparation. *Bar* 50 μm.

Fig. 2. OX6-immunoreactivities in control (**a**) and injured teeth (**b–f**). **a.** OX6-immunopositive cells are located predominantly beneath the odontoblast layer and around blood vessels in the dental pulp. Most of the immunopositive cells display a dendritic appearance. **b.** Immediately after operation. OX6-immunopositive cells appearing in dendritic fashion shift inwards together with the separated odontoblasts. **c.** 6 hours after operation. OX6-immunopositive cells have moved in the vicinity of the predentin. Some of them appear to extend their processes toward the dentinal tubules (*arrows*). **d.** 12 hours after operation. OX6-immunopositive cells accumulate along the pulp-dentin border and extend their processes deep into the dentinal tubules (*arrows*). **e.** 24 hours after operation. OX6-immunopositive cells with their processes remain along the pulp-dentin border (*arrows*). **f.** 72 hours after operation. OX6-immunopositive cells are located beneath the differentiated odontoblast layer. *D* dentin, *DP* dental pulp, *OB* odontoblasts, * exudative lesion enhanced artificially during tissue preparation. *Bar* 50 μm.

Fig. 3. Electron micrographs of OX6-immunopositive cells beneath the odontoblast layer in the control tooth. **a.** Immunoreactive products

for Ia antigen are observed as electron-dense deposits in the cell membrane of an immunopositive cell. However, the odontoblasts (*OB*) do not exhibit any immunoreactions. **b.** Higher magnification of the boxed area in **a**. The immunopositive cell possesses a well-developed Golgi apparatus, mitochondria, tubulovesicular structures (*arrowhead*) and multivesicular bodies (*arrow*) but no phagosomes in its cytoplasm. *D* dentin, *DP* dental pulp. *Bar* 5 μm (**a**), 3 μm (**b**).

Fig. 4. Electron micrograph of OX6-immunopositive cells at the pulp-dentin border in an injured tooth after 6 hours. Cell debris comprising degenerated odontoblasts and a vesicle-appearance (*) are observed in the dentinal tubules. Both OX6-positive (*arrow*) and negative cells (*arrowheads*), which include numerous phagosomes, are arranged along the pulp-dentin border. *PD* predentin, *DP* dental pulp. *Bar* 4 μm.

Fig. 5. Electron micrograph of OX6-immunopositive cells at the pulp-dentin border in the injured tooth after 12 hours. The immunopositive cells extending cytoplasmic processes in the dentinal tubules contain characteristic tubulovesicular structures (*arrow*), multivesicular bodies (*arrowheads*) and vacuoles (*). *D* dentin, *DP* dental pulp. *Bar* 3 μm.

Fig. 6. Electron micrographs of OX6-immunopositive cells beneath the odontoblast layer in the injured tooth after 72 hours. **a.** The differentiated odontoblasts develop a Golgi apparatus and rough-surfaced endoplasmic reticulum in their cytoplasm. Note the presence of an immunopositive cell beneath the odontoblasts (*OB*). **b.** Higher magnification of the boxed area in **a**. An immunopositive cell possesses a well-developed Golgi apparatus, mitochondria, tubulovesicular structures, and multivesicular bodies (*arrow*). Bars 5 μm (**a**), 3 μm (**b**).

Fig. 7. A schematic diagram summarizing the spatiotemporal relationship between Hsp 25-immunopositive cells (*opaque*) and class II MHC-positive cells including dendritic cells (*opaque & DC*) and macrophages (*opaque & Mc*) following cavity preparation. In a control tooth, a class II MHC-positive dendritic cell is located beneath the Hsp 25-immunoreactive odontoblast cell layer. Class II MHC-positive and negative macrophages, and a polymorphonuclear leukocyte (*PML*) gather at the pulp-dentin border 6 hours after cavity preparation. A class II MHC-positive dendritic cell appears along the pulp-dentin border and extends its processes into the exposed dentinal tubules during postoperative 12-24 hours. By postoperative 72 hours, a class II MHC-positive dendritic cell moves beneath newly-differentiated odontoblasts with Hsp 25-immunoreactivity to

locate at the pulp-dentin border. *CL* capillary lumen, *MC* mesenchymal cells, *OB* odontoblasts.