An immunocytochemical study of pulpal responses to cavity preparation by laser ablation in rat molars using antibodies to heat shock protein (Hsp) 25 and class II MHC antigen

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An immunocytochemical study of pulpal responses to cavity preparation by laser ablation in rat molars using antibodies to heat shock protein (Hsp) 25 and class II MHC antigen

Abstract Initial responses of odontoblasts and immunocompetent cells to cavity preparation by laser ablation were investigated in rat molars. In untreated control teeth, an intense heat shock protein (Hsp) 25-immunoreactivity was found in the cell bodies of odontoblasts, whereas cells immunopositive for the class II major histocompatibility complex (MHC) antigen were predominantly located beneath the odontoblast layer in the dental pulp. Cavity preparation caused the destruction of the odontoblast layer and the shift of most class II MHC-positive cells from the pulp-dentin border toward the pulp core at the affected site. Twelve h 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, but subsequently disappeared from the pulp-dentin border together with Hsp 25-immunopositive cells by 24 h

after the operation. By postoperative 3–5 d, a distinct abscess formation consisting of polymorphonuclear leukocytes was found in the dental pulp. The penetration of masses of oral bacteria was recognizable in the dentinal tubules beneath the prepared cavity. These findings indicate that cavity preparation by laser ablation induced a remarkable inflammation by continuous bacterial infections via dentinal tubules in this experimental model that delayed the pulpal regeneration.

Key words cavity preparation; heat shock protein; immunocompetent cell; laser ablation; odontoblast.

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Introduction

Laser appliances with various wavelengths have been applied to cavity preparation in clinical dentistry (Hibst and Keller 1989; Matsumoto et al. 1996; Aoki et al. 1998). In particular, erbium: yttrium-aluminum-garnet (Er:YAG); erbium, chromium: yttrium-scandium-gallium-garnet (Er,Cr:YSGG); and chromium, thulium, erbium: yttrium-aluminum-garnet (CrTmEr:YAG) lasers with similar individual wavelengths (2.94 μm, 2.79 μm and 2.69 μm, respectively) have been utilized as laser appliances for ablating teeth because of an absorption spectrum close to that of water (Hoke et al. 1990; Keller and Hibst 1997; Eversole et al. 1997; Hossain et al. 1999; 2001). In general, these appliances are advantageous for allowing comparatively soundless, minimal vibration and inhibition of heating compared with conventional drilling methods.

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 (Ciocca et al. 1993; Arrigo and Préville 1999). This protein has been reported to possess diverse functions (Huot et al. 1991; Jakob et al. 1993;

Lavoie et al. 1993a, 1993b; Mehlen et al. 1995, 1996, 1997; Mairesse et al. 1996; Wu and Welsh 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 in intact teeth (Ohshima et al. 2000, 2002) and under experimental conditions (Ohshima et al. 2001a, 2001b, 2003), suggesting that this protein is a useful marker for the differentiation of odontoblasts during the pulpal healing process.

On the other hand, the dental pulp is known to contain many immunocompetent cells that serve in the initial defense reaction and antigen-presentation. The most abundant immunocompetent cells in the dental pulp are those cells with the class II major histocompatibility complex (MHC) antigen. In rats, the class II MHC antigen corresponds to the Ia antigen, which can be recognized by an OX6 antibody (McMaster and Williams 1979). These OX6-immunopositive 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) and carious teeth (Yoshiba et al. 1996; Izumi et al. 1996; Kamal et al. 1997; Sakurai et al. 1999).

To date, pulpal reactions to cavity preparation with laser ablation have been reported to be almost identical to those with ordinary high-speed drills such as an air turbine (Dostálová et al. 1997; Takamori 2000; Tanabe et al., 2002). We have recently investigated the pulpal responses of the dental pulp to cavity preparation with conventional drilling by immunocytochemistry for Hsp 25 (Ohshima et al. 2001b, 2003) and class II MHC antigen (Ohshima et al. 1995, 2003) in rat molar teeth. In these studies, we were able to demonstrate the differentiation process of odontoblasts and the drastic responses of immunocompetent cells during the pulpal healing process incident to cavity preparation. However, in contrast to numerous studies on pulpal responses after cavity preparation with ordinary drilling (Smith, 2002), information on those with laser ablation is meager. In particular, the pulp regeneration processes during cellular events following laser ablation remain to be fully understood. Furthermore, to our knowledge, there has been no available data regarding

the responses of the pulp tissue to cavity preparation with CrTmEr:YAG laser, except for a single report on the surface condition of the prepared cavity (Kermani et al. 1993). The present study was, therefore, undertaken to examine the initial responses of odontoblasts and immunocompetent cells to cavity preparation using CrTmEr:YAG laser in rat molars by immunocytochemistry for Hsp 25 and the class II MHC antigen.

Materials and methods

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

Thirty-nine Wistar rats, 100 d old, were used in this study. Under anesthesia by an intraperitoneal injection of chloral hydrate (350 mg/kg), a groove-shaped cavity (the width of the remaining dentin being around 50–150 μm) was prepared on the medial surface of the upper right first molar by laser irradiation under water-cooling. The laser used in this experiment was a pulsed CrTmEr:YAG laser (wavelength is 2.69 μm; Niic Co. Ltd., Niigata, Japan). The laser parameter was around 200–250 mJ/pulse and 5 pulse/s. The cavity received no further treatment such as air drying, etching, or filling except for one group where the cavity was sealed with filling materials. The upper left first molar of the same animal was used as a control.

Materials were collected in groups of six animals at intervals of 0, 6, 12 and 24 h, and 3 and 5 d after cavity preparation for opened cavity experiment, because we have already observed that dynamic pulpal responses occurred at these stages in the previous reports (Ohshima et al.,

1995, 2001b, 2003). All six animals showed almost the same pulpal reactions to laser ablation. Furthermore, the cavity was sealed with grass ionomer cement at day 3 in three animals. 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 10% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution for 4 wk 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.

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 immunoreaction.

Further characterization of the primary antibody has been reported elsewhere (Plumier et al. 1997; Ohshima et al. 2000; Shimizu et al., 2000).

Results

Controls

Hsp 25-immunohistochemistry in the dental pulp demonstrated an intense immunoreaction in the odontoblasts, but a weak one in other cellular elements including nerve fibers and blood vessels (Fig. 1a). The immunoreaction within odontoblasts was observed in the cell bodies and cell processes limited within the predentin. No immunonegative odontoblast was found in the control teeth (Fig 1b). Many OX6-immunopositive cells were widely distributed throughout the dental pulp of rat molars, predominantly at the periphery of the pulp tissue (Fig 1c). Most of the subodontoblastic immunopositive cells displayed dendritic profiles, and some of them extended their processes into the odontoblast layer (Fig. 1d). The pulpo-dentinal border zone including the odontoblast cell layer contained few OX6-positive cells in the untreated control teeth.

Immediately after cavity preparation without filling material

Cavity preparation rapidly induced an exudative lesion between the predentin and damaged odontoblast layer (Fig. 1e, g). The odontoblasts under the prepared cavity suffered severe damage; many odontoblasts at the affected site lost the Hsp 25-immunoreaction (Fig. 1e). However, Hsp 25-positive cells either with slender cell processes or round positive cells without apparent cell processes, intermingled at the damaged odontoblast cell layer (Fig. 1f). The OX6-immunopositive cells showing a dendritic appearance at the affected site shifted inwards together with the separated and damaged odontoblasts (Fig. 1g, h).

Six h after cavity preparation without filling material

The apparent exudative lesion had disappeared by 6 h (Fig. 2a, c). The damaged odontoblasts with Hsp 25-immunoreaction came to show round profile in the impaired odontoblast layer (Fig. 2b). A part of the predentin contained cell debris positive for Hsp 25 (Fig. 2b). However, the intact odontoblasts did not show any changes in immunoreaction, shape, or arrangement (Fig. 2a). In OX6-immunohistochemistry, most positive

cells, round in shape, migrated to align themselves at the pulp-dentin border (Fig. 2c, d).

Twelve h after cavity preparation without filling material

The damaged odontoblast layer had fewer Hsp 25-immunopositive cells than that at the previous stage (Fig. 2e). The damaged odontoblast layer appeared to decrease in immuno-intensity. However, some Hsp 25-positive round cells at the periphery of the damaged odontoblast layer had slender cytoplasmic processes (Fig. 2f). In contrast, the OX6-immunopositive cells accumulated at the affected area along the pulp-dentin border (Fig. 2g); they frequently extended their processes deep into the dentinal tubules (Fig. 2g, h). On the other hand, the distribution pattern and shape of OX6-positive cells at the other intact site was the same as that at the previous stage.

Twenty-four h after cavity preparation without filling material

Many inflammatory cells intensely stained with methylene blue occurred between the predentin and the damaged odontoblast layer (Fig. 3a). The

Hsp 25-immunoreactive cells showed a single-layered arrangement, but such positive cells without their cell processes were very scarce (Fig. 3b). OX6-immunohistochemistry demonstrated a shift of the positive cells from the pulp-dentin border to a location around the inflammatory region (Fig. 3c, d). These positive cells completely lacked any elongated processes, and appeared rounded (Fig. 3d). No OX6-immunoreaction was recognized in the predentin or dentin at the inflammatory region.

Three to 5 d after cavity preparation without filling material

By 3 d after cavity preparation, the inflammatory region had expanded rapidly to form an abscess in the dental pulp restricted to the affected site (Fig. 3e, g). No Hsp 25-positive cells were discernible inside the inflammatory abscess, but comparatively flat cells with Hsp 25-immunoreaction were arranged around this focus (Fig. 3f). The OX6-immunopositive cells were also scattered in the dental pulp, these being located far from the inflammatory abscess (Fig. 3g). At this stage, methylene blue-stained substances were often found in the dentinal tubules

associated with such lesions (Fig. 3h). At higher magnification, they appeared as masses of microorganisms (Fig 3h, inset).

Three d after cavity preparation with filling material

The immunoreactivity for Hsp 25 was not recognizable beneath the damaged dentin, whereas the intact odontoblasts show an intense immunoreaction. A weak immunoreaction for Hsp 25 was detected in the nervous and vascular elements, and no abscess formation was observed in the dental pulp (Fig. 4a, b). OX6-immunopositive cells were scattered in the pulp, and some were situated at the affected area along the pulp-dentin border although the extension of cellular processes into the dentinal tubules was slight in contrast with the group without filling material at postoperative 12 h (Fig. 4c, d).

Discussion

By use of the antibodies to Hsp 25 and class II MHC antigen, the present immunocytochemical study was able to demonstrate clearly the pulpal reaction to cavity preparation by laser ablation in rat molars.

The pulpal responses to laser ablation as shown in this study were far exceeded our expectations. Our most noteworthy finding is that laser ablation induced an easy infiltration of PMLs to form an abscess lesion in the dental pulp, something which never has been reported in previous experimental studies using other laser appliances such as Er:YAG and Er,Cr:YSGG (Dostálová et al. 1997; Takamori et al. 2000; Tanabe et al., 2002) and tooth grinding by air turbine (Ohshima et al. 1990, 1995, 2001b, 2003). This difference in tissue reaction between drill- and laser-prepared cavities may be explained by the surface condition of the cavity. The drilling essentially forms a smear layer at the surface of the prepared cavity, in contrast to none in the laser-prepared cavity (Hossain et al. 2001, Takeda et al. 1998; Yamada et al. 2001). Since the smear layer plugs the exposed dentinal tubules to prevent the invasion of bacteria into the dental pulp (Love et al. 1996; Peters et al. 2000), it is reasonable to consider that the

bacterial infection caused the abscess formation. This idea is strongly supported by the present findings that the deep penetration of bacteria-like structures into the dentinal tubules was found at postoperative 3–5 d. On the other hand, the depths of the cavity (a deep cavity in this study versus a comparatively shallow cavity in the previous papers) may account for the different tissue responses between the types of laser appliances (Er:YAG, Er,Cr:YSGG and CrTmEr:YAG lasers) with laser parameters similar to those in the previous experiments (Matsumoto et al. 1996; Takizawa et al. 1996). To date, the abscess formation has been reported in canine teeth as a case of failure when the cavity floor was close to the dental pulp (Takizawa et al. 1996). Furthermore, we can consider the status of the prepared cavities: the prepared cavities were left without any treatment in this study while they were filled with glass ionomer cement and/or adhesive resin in the previous studies (Takizawa 1996; Takamori 2000). It is likely that the unexpected abscess formation was caused by different experimental designs between the previous (Takizawa 1996; Takamori 2000; Tanabe et al., 2002) and present studies. In the additional experiment (Fig. 4), actually, the sealing of the prepared cavity with filling

material prevented the bacterial infection through the dentinal tubules to induce no abscess formation in this study. The easy infiltration of PMLs to form an abscess lesion may not be due to the laser ablation but be bacterial infection due to exposed orifices of dentinal tubules. A complete and more careful sealing of the prepared cavity after laser ablation is required to prevent abscess formation.

Cavity preparation caused an edematous reaction between the injured odontoblasts and predentin, and the immunoreactivity for Hsp 25 was decreased in intensity in the odontoblasts. Our previous studies demonstrated that conventional cavity preparation caused the destruction of the odontoblast layer to form an edematous lesion, some damaged odontoblasts retained the immunoreactivity for Hsp 25 (Ohshima et al., 2001b, 2003) as well as the results obtained in the current study. The findings indicate that most odontoblasts degenerate, but a part of them survive against artificial external stimuli. However, there is no evidence as to whether the down-regulated transcription of Hsp 25 occurs in the injured odontoblasts or not. Further study to investigate the mRNA

expression of Hsp 25 using *in situ* hybridization is needed to clarify the functional significance of altered expressions of Hsp 25 in the odontoblasts.

It is interesting that the laser ablation induced different reactions between the OX6-positive immunocompetent cells and PMLs in this study. At the initial stage, the OX6-positive cells were temporarily arranged along the pulp-dentin border and extended their cytoplasmic processes into the dentinal tubules, consistent with the observations of previous experimental studies (Ohshima et al. 1995, 2003). However, the cells disappeared from the pulp-dentin border at postoperative 24 h. In general, the class II MHC-positive cells (OX6-positive cells in rats) participate in the initial immune-response to serve as an antigen-presenting cell (Steinman 1991). On the other hand, increased PMLs response with phagocytotic activity may well be due to bacterial infection after cavity preparation. Their responses shown in this study well reflect the different functions between the OX6-positive cells and PMLs.

Another notable finding is the arrangement of the Hsp 25-immunopositive cells around the abscess lesion. However, the laser-prepared cavity showed delayed pulpal healing in this study in

comparison with the experimental data on conventional cavity preparation (Ohshima et al. 1990, 1995, 2001b, 2003). This delayed healing was observed even in a case of sealing with glass ionomer cement in the current results. In our previous studies on the regeneration of odontoblasts in rat molars (Ohshima et al. 2001b, 2003), the expression of Hsp 25-immunoreactivity has been suggested as a useful marker for newly-differentiated odontoblasts. Furthermore, our preliminary examinations demonstrated that the matrix was deposited around the abscess lesion after 10-20 d in the same experimental model used in this study (unpublished findings). These findings indicate the possibility that the Hsp 25-immunoreactive cells in question are mesenchymal cells with the potential for differentiating into odontoblasts. Thus, the effects of bacterial infection and subsequent abscess formation seem to have delayed pulp regeneration.

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Figure legends

Fig. 1. Hsp 25- (a, b, e, f) and OX6-immunoreactivities (c, d, g, h) in the control (a–d) and injured teeth after 0 h (e–h). a. Intense immunoreaction for Hsp 25 is found in the odontoblasts. Some nerve fibers and blood vessels show a weak Hsp 25-immunoreaction (*arrowhead*). b. Higher magnification of the boxed area in a. Odontoblasts in the coronal pulp are intensely immunoreactive for Hsp 25 in their cytoplasm, especially in the medial and distal cytoplasm. Their cell processes in the predentin are seen to be immunoreactive. c. OX6-immunopositive cells are predominantly located at the periphery of the pulp tissue. d. Higher magnification of the boxed area in c. Most of the subodontoblastic immunopositive cells appear a dendritic shape, and some extend their processes into the odontoblast layer (*arrowheads*). e. The odontoblasts under the prepared cavity suffer severe damage, and exudative lesions occur between the predentin and damaged odontoblast layer. f. Higher magnification of the boxed area in e. The cells, round in shape without cell processes, retain the Hsp 25-immunoreactivity (*arrow*s). g.

OX6-immunopositive cells shift inwards together with the separated odontoblasts. h. Higher magnification of the boxed area in g. OX6-immunopositive cells still display a dendritic appearance at this region. *C* prepared cavity, *D* dentin, *DP* dental pulp, *OB* odontoblasts, ∗ artificial gap.

Fig. 2. Hsp 25- (a, b, e, f) and OX6-immunoreactivities (c, d, g, h) in the injured teeth after 6 (a–d) and 12 h (e–h). a. Exudative lesions have disappeared by 6 h. A part of the predentin contains cell debris positive for Hsp 25. b. Higher magnification of the boxed area in a. Hsp 25-immunoreactivity remains in the impaired odontoblasts with round profiles. c. OX6-immunopositive cells have moved to a location in the vicinity of the predentin. d. Higher magnification of the boxed area in c. Most immunoreactive cells appear round in shape, and some extend their processes into the dentinal tubules (*arrowhead*). e. The damaged odontoblast layer includes fewer Hsp 25-immunopositive cells than that at the previous stage. f. Higher magnification of the box area in e. The Hsp 25-immunoreactive cells

with slender cytoplasmic processes are discernible beneath the degenerated odontoblast layer (*arrows*). g. OX6-immunopositive cells accumulate along the pulp-dentin border. h. Higher magnification of the boxed area in g. OX6-immunopositive cells extend their processes deep into the dentinal tubules (*arrows*). *C* prepared cavity, *D* dentin, *DP* dental pulp, *OB* odontoblasts.

Fig. 3. Hsp 25- (a, b, e, f) and OX6-immunoreactivities (c, d, g, h) in the injured teeth after 24 h (a–d) and 3–5 d (e–h). a. No apparent immunoreaction for Hsp 25 is found in the pulp-dentin border. b. Higher magnification of the boxed area in a. Many inflammatory cells intensely stained with methylene blue are discernible between the predentin and damaged odontoblast layer. c. OX6-immunopositive cells are located apart from the pulp-dentin border. d. Higher magnification of the boxed area in c. Immunopositive cells appear round in shape (*arrowhead*). e. An inflammatory lesion without immunoreactivity for Hsp 25 (∗) is formed beneath the prepared cavity in the dental pulp. f. Higher magnification of the boxed area in e.

The lesion is surrounded by Hsp 25-immunopositive cells (*arrows*) that are never seen at the pulp-dentin border in the injured area. g. OX6-immunopositive cells are scattered in the dental pulp, but never inside the lesion. h. Masses of oral bacteria stained with methylene blue are recognized in the dentinal tubules associated with the lesion. Inset. Higher magnification of the boxed area in h. Round masses of bacteria-like structures are found in the dentinal tubules. *C* prepared cavity, *D* dentin, *DP* dental pulp, *OB* odontoblasts.

Fig. 4. Hsp 25- (a, b) and OX6-immunoreactivities (c, d) in the injured teeth with filling material after 3 d. a. No apparent immunoreaction for Hsp 25 is found in the pulp-dentin border in the suffered area, whereas the intact odontoblasts show an intense immunoreaction. b. Higher magnification of the boxed area in a. The nervous and vascular elements (*arrows*) show a weak Hsp 25-immunoreaction. c. OX6-immunopositive cells are scattered in the dental pulp, and some are located along the pulp-dentin border. d. Higher magnification of the boxed area in c. Immunopositive cells extend their cellular

processes into the dentinal tubules although their extension is slight (*arrowheads*). *C* prepared cavity, *D* dentin, *DP* dental pulp, *OB* odontoblasts.